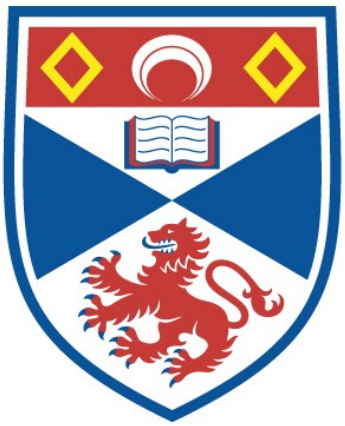
Investigating the role of harmful environmental organisms in multifactorial gill pathology in salmonids

Morag Clinton

A thesis submitted for the degree of PhD
at the
University of St Andrews



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Salmonid Gill Health

To my family,
original and acquired

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List of Abbreviations

AGD	Amoebic Gill Disease
AK	Anna Kintner
AW	Adam Wyness
BH	Benjamin-Hochberg
CGD	Complex Gill Disease
CO_2	Carbon Dioxide
EK	Elzbieta B Krol
FISH	Fluorescence *in situ* hybridisation
GIALT	Gill-Associated Lymphoid Tissue
GIT	Gastrointestinal Tract
GO	Gene Ontology
H_2O_2	Hydrogen Peroxide
HAB	Harmful Algal Bloom
HGNC	HUGO Gene Nomenclature Committee
IHN	Infectious hematopoietic necrosis
ILT	Inter-branchial Lymphoid Tissue
KEGG	Kyoto Encyclopaedia of Genes and Genomes
q-PCR	Quantitative Polymerase Chain Reaction
PCA	Principal Component Analysis
PCoA	Principal Coordinate Analysis
PCR	Polymerase Chain Reaction
PGD	Proliferative Gill Disease
PGI	Proliferative Gill Inflammation
RDA	Redundancy analysis
ROS	Reactive Oxygen Species
SDEG	Significantly Differently Expressed Genes
SSF	Scottish Sea Farms

Author Contributions

Work as part of this thesis could not have been conducted without the assistance and previously work of several contributors.

- Chapter 3 was performed using archived material from previous PhD student Anna Kintner. Anna also provided insight into her culture conditions and the ecological significance of cnidarians sampled in this work for publication.
- Chapter 4 was performed as part of a collaboration with Aarhus University in Denmark, where challenge trials were conducted as part of the HAB project. Laboratory training and assistance was provided by Dr Elzbieta B Krol at the University of Aberdeen, where experimental work was performed.
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Publications

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Abstract

In this thesis, histopathology and molecular research tools were utilised to investigate gill health in salmonids. Of interest were the roles of the harmful environmental organism's cnidarian jellyfish and toxic phytoplankton in gill pathologies of farmed fish. Multifactorial (complex) gill diseases are poorly understood disorders of fish and problematic for aquaculture, particularly in the marine environment. This thesis attempts to address the involvement of several factors in initiation and potentiation of altered health states within gills, to enhance the collective understanding of gill disease in salmonids. Results chapters address the question of the involvement of these harmful environmental organisms in gill disease and explore the microbial communities of salmonid gills with the aim of understanding the process of dysbiosis. Chapters explore the microbiomes of cnidarian jellyfish for microbial pathogens that might be transmitted, the gill transcriptome response to toxic phytoplankton exposure, and alterations to the gill microbiome with histopathological change.

The work of this thesis identified the presence of potentially harmful microbes within the microbiome of sampled cnidarian jellyfish. Alongside the previously demonstrated ability of cnidarian organisms to elicit traumatic damage to fish, it seems vector transmission might represent an additional avenue of harm through introduction of bacterial agents. The transcriptomic response of gills to phytoplankton was also explored, representing the first study of altered genetic expression of fish gills in response to toxin production phytoplankton. Results provide useful insight into the mechanisms of tissue damage by phytoplankton *Prymesium parvum,* as well as potentially informing future mitigation strategies for algal blooms through study of the pathways of tissue response. Final chapters of this thesis provide insight into the microbial community structure of farmed Atlantic salmon and factors influencing altered or 'dysbiotic' microbial populations. Temporal and pathology-associated alterations were observed within gill microbial communities in a farmed situation, as well as the impact of on-farm activities such as hydrogen peroxide treatment. Significant variation in results were identified with varied sampling methodology, highlighting the requirement for careful consideration of experimental design in the

study of the microbiome, as well as providing early insight into the potential for niche partitioning of the gill microbiota.

Overall, results of this thesis emphasise the potential for harm from cnidarian jellyfish and phytoplankton in both farmed and wild fisheries. The gills are a delicate organ constantly exposed to environmental insult that suffer complex, mixed aetiology disorders as a result. These disorders are problematic to diagnose and treat, and prevention therefore seems preferable to cure. Results identify the potential initiating and propagating role of various factors in gill disorders, as well as exploring additional avenues of health status monitoring. The final chapter of this thesis discusses the main findings of this research and the potential applications both in further study and directly to enhance the aquaculture production of salmonids.

Chapter 1

1. General Introduction

For many years, researchers have been interested in the anatomy and function of gills, a respiratory organ that allows fish to survive underwater. Their research has shown that in addition to respiration, gills perform additional essential functions, including in adaptation to novel or hostile environmental conditions. Gills are an important organ therefore in the physiological regulation and adaptation of fish, experiencing unique challenges due to their front-facing nature and exposure to the environment. The external location of gills makes them susceptible to trauma and infectious disease, particularly in contrast to organs maintained within sterile body cavities. For this very reason, they provide a fascinating topic for the study of disease. No such purely external organ with essential function exists in humans, where the skin acts as a thick protective mechanism. Gills perform their essential functions in gas exchange and osmoregulation whilst in direct contact with the aquatic environment. Gill disease is therefore a concern in all fish, however it appears particularly problematic in the aquaculture industry, where fish such as salmonids and carp are maintained in artificial conditions that can predispose to pathologies. Salmonids are teleost finfish, adapted for both freshwater and marine environments and are considered to be one of the most commercially important aquaculture-produced fish species in the world. The study of gill health in salmonids is therefore an important field of research for both wild fisheries and aquaculture systems.

The response of gills to many harmful agents is as yet poorly understood. As physical barriers that separate fish from the external environment, gills must possess a varied repertoire of defences against infectious microbes and stressors. The impact of individual directly infectious agents of disease such as bacteria and viruses has been a key area of research in fish health (Bruno et al., 2013), even more so since the advent of large-scale aquaculture production. Enclosure of fish presents an unnatural environment where external variables that might be of little concern to wild counterparts can become problematic for intensively produced fish. Non-infectious organisms such as phytoplankton and cnidarian jellyfish have appeared in the news with increasing frequency due to their association with large-scale mortalities of fish in both farmed and

wild environments (Bosch-Belmar et al., 2017b; Purcell et al., 2007; The Scottish Government, 2006). Recent research has demonstrated that these organisms are capable of eliciting harm in fish, however the mechanisms of this impact and the role of these organisms in commonly observed farmed fish pathology are still poorly understood.

1.1 Aquaculture production

1.1.1 Global aquaculture and important production species

Aquaculture is a diverse and growing global industry, although relatively new in comparison with terrestrial livestock production. Both marine and freshwater farming are present in many countries all over the world, for example for production of crustaceans, molluscs and fish. Cyprinidae species account for the greatest cultivated tonnage globally, farmed in freshwater ponds particularly in Asia (FAO, 2018), but Salmonidae fetch a higher price per kilogram, making the aquaculture industry for the production of these fish one of the most valuable globally (FAO, 2016a). North American aquaculture is dominated by finfish production, particularly Siluriformes (catfish) and Salmonidae (salmonids) (Harvey et al., 2017), while aquaculture in Europe is focused in particular on production of the salmonid species *Salmo salar* (Atlantic salmon) (Clark and Bostock, 2017). The greatest outputs of Atlantic salmon are from Chile, Norway, Tasmania, Scotland, British Columbia and the Northern United States (FAO, 2016a). Smaller scale production occurs in many more countries too, including Ireland, France, New Zealand and Spain (Graziano da Silva, 2016).

Carp species like *Ctenopharyngodon idella* (grass carp) are a popular aquaculture fish in Asian countries, but Cyprinids like carp have a variety of uses. *Cyprinus rubrofuscus* (koi carp) are valuable ornamental fish, whereas *Cyprinus carpio* (common carp) are a popular species for stocking ponds for sport fishing in the United Kingdom. The Cichilid *Oreochromis niloticus* (Nile tilapia) is another important aquaculture species, more frequently farmed in the east and Africa.

All these fish can suffer from gill diseases, however agents and severity of disease vary with production species, as do methods and environments of production. Salmonids for

example are generally raised in a controlled recirculating system during the freshwater stage of their lifecycle, and then in the case of Atlantic salmon, are transferred following smoltification to open net pens within the marine environment, to grow for another 1-2 years prior to harvest (**Figure 1.1**). Atlantic salmon are by no means the only commercially important salmonid though. *Oncorhynchus mykiss* (rainbow trout) are reared with varied methodologies, including production using similar sea-farming systems to Atlantic salmon or in pond and raceway systems (Balseiro et al., 2018; Bosakowski and Wagner, 1995). The health of these fish is of interest with regards to optimising their aquaculture production, as well as conservation of various wild sub-species. In North America, Pacific salmon including *Oncorhynchus gorbuscha* (pink salmon) and *Oncorhynchus keta* (chum salmon) are reared through the 'ocean ranching' system of hatchery production and release, for bolstering of coastal fisheries (Evenson et al., 2018). This system of production is popular in the USA and Japan, whereas Canada favours farming of Atlantic salmon. Iceland practices ocean ranching too, but for Atlantic salmon production.

In Scotland, fish farming began in the 1960s small scale as part of crofting. Now, the Scottish industry produces over 179,000 tonnes of Atlantic salmon annually, with a farm-gate value in excess of £633 million in 2013 (Munro and Wallace, 2015; Scottish Salmon Producers Organisation, 2014). A total of £944 million worth of fish and seafood products were exported by Scotland in 2017, a figure over five times the value of Scottish meat, dairy, eggs and live animal exports combined ("Region trade information, HM Revenue and Customs," 2019). Production of Atlantic salmon accounts for 95% of aquaculture in Scotland, and rainbow trout production does account for 12% of seafood production in the UK as a whole (Clark and Bostock, 2017). Clearly then, production of farmed finfish, particularly salmonids, is an important and expanding industry. With declining wild fish catches and increasing pressure on food security from a growing global population, demand for the products of aquaculture looks set to continue to increase (Duarte et al., 2009; ICES Secretariat, 2015; The World Bank, 2013).

1.1.2 Challenges to fish health during production

As well as being potentially the most important food production industry in many countries including Scotland, aquaculture production of salmonids is a comparatively new industry, and so does not benefit from the background of historical research that informs much of terrestrial livestock production. As such, it is a prioritized and exciting field of research for specialists in many fields, including veterinarians, who seek to maximize animal welfare as well as industry production (BBSRC, 2014; MGSA Science & Research Working Group, 2014; Scottish Aquaculture Innovation Centre (SAIC), 2015).

Like all livestock production industries, intensive aquaculture faces many infectious and non-infectious challenges to animal health. Different systems of production experience different challenges to fish health and welfare. Disease is currently considered the primary constraint on industry growth in Atlantic salmon production, impacting not only financial output, but also the health and welfare of fish (Lafferty et al., 2015; Subasinghe, 2005). Disease limits production in other aquaculture systems too of course, the viral condition infectious hematopoietic necrosis (IHN) for example is largely blamed for the altered trend in hatchery production in Alaska, with reduced production of sockeye and increased production of the less susceptible but also less lucrative pink and chum salmon species (NOAA, 2018). Gill disease is frequently reported in carp as well, with mixed parasitic infections. Carp are however considered fairly robust, particularly in the context of gill disease. Unlike carp, salmonids lack the ability to remodel gill tissue in response to varied disease states (Matey et al., 2008) or hypoxic conditions (Nilsson et al., 2012). With a high oxygen demand (Remen, 2012), salmon do not compensate as well during gill pathology.

Figure 1.1 Aquaculture production of Atlantic salmon

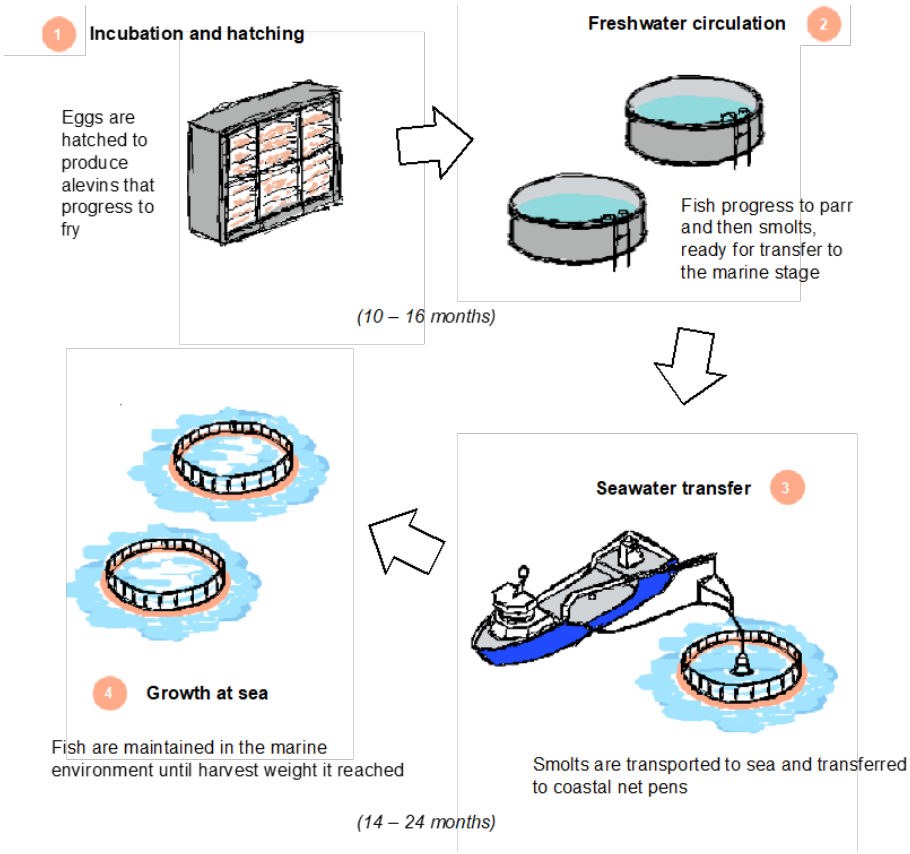


***Figure 1.1**: Average production cycle of Atlantic salmon, from incubation to harvest at approximately 4kg. Whilst a number of different production methods exists for the various salmon species, this method of production is most frequently employed for Atlantic salmon. Atlantic salmon represent the most commonly farmed species of the Salmonidae and are documented as experiencing pathologies at the marine stage.*

Whilst fish reared entirely through aquaculture benefit from vaccinations, disease-specific functional feeds and pharmaceutical therapies, the high stocking density and intensive production of these animals mean they suffer many health conditions less frequently observed in their wild counterparts. During production of Atlantic salmon, fish are particularly prone to disease on introduction to the marine environment for maricultural (Speare, 2007), due at least in part to the stress of transfer, but also because of exposure to new, unfamiliar pathogens (Jeffries et al., 2014). Hatching eggs through to production of parr is performed in relatively controlled freshwater environments,

often with recirculating systems that limit exposure to many potential pathogens. Whilst saline recirculating systems do exist, the norm in Scotland is for fish to be moved from fresh water to open sea cages following smoltification, where they are maintained in static net systems. (Munro and Wallace, 2015; Speare, 2007). In the marine environment, fish are exposed to a different mix of infectious challenge. Isolation from potentially pathogenic organisms and control of environmental conditions is also highly difficult, especially in the close confines of intensively stocked cage systems, making the marine phase of production arguably the most important area of focus for disease prevention (Conte, 2004; Rodger, 2007).

Although treatment for external parasites represents a large financial outlay for the industry (Pike et al., 1999), as well as being a focus for the media due to their reported exchange with wild fish (Nekouei et al., 2018), endemic gill disease in Atlantic salmon appears to be associated with a greater number of direct mortalities (Rodger et al., 2011), and might therefore be having a comparable or even greater impact on the production performance of stock. Gill conditions are estimated to be the largest cause of mortalities in marine stocks, with few fish observed to be without pathology following introduction to seawater. As much as a 12% total loss of total stock in the Irish salmon industry between 2003-2006 was attributed to gill disease (Mitchell et al., 2011b; Rodger, 2007). For these reasons, much research into the gill pathologies of salmonids is therefore conducted with a focus on farmed salmonids.

Gill pathologies are of course not exclusive to farmed salmonids. Studies of wild *Oncorhynchus tshawytscha* (Chinook salmon) suggest many gill pathogens are encountered in the marine environment (Van Gaest et al., 2011), but monitoring and an understanding of the impact of these diseases on fish is problematic to assess while they are swimming free in the ocean (Jeffries et al., 2014). The practice of fish farming through containment of Atlantic salmon in caged systems throughout the production cycle is more easily accessed for the assessment of gill disease in fish, by making it much easier to obtain samples. The intensive method of production of Atlantic salmon also appears to predispose fish to disease, with gill pathologies frequently observed within stock (Bruno et al., 2013; Rodger et al., 2011). High stocking density and other

variables encountered by fish in aquaculture production appear to undermine fish health and reduce resistance to disease (Arkoosh et al., 1998). Stressful conditions and intercurrent disease experienced in aquaculture production are just two of many demonstrated causes of reduced host resistance (Black and Pickering, 1998) that likely exacerbate gill disease in farmed fish. It is now well-recognized that teleost fish such as salmon experience pain and react with both physiological and behavioural stress responses to noxious stimuli (Chandroo et al., 2004; Conte, 2004; Sneddon, 2003). Stress in fish manifests in many ways, with hormonal and immunological changes in fish that utilise energy and can reduce the efficacy of defences (Schreck and Tort, 2016). Reducing disease incidence is therefore considered a crucial step in safeguarding the welfare of farmed fish (RSCPA, 2015). Impaired health in fish causes reduced feeding as well as diverting energy from growth and production (Barton, 2002; Black and Pickering, 1998; Harper and Wolf, 2009), with consequent negative impacts on economic output of the industry. Treatment of disease is also currently one of the biggest financial outlays of the aquaculture industry, second only to feed purchase (Assefa and Abunna, 2018; Black and Hughes, 2017; Love et al., 2017). All this leads then to the reduced ability of producers to generate revenue from their stock, with longer production times and increased costs. Understanding the pathology of these conditions and minimising their incidence is therefore in the interests of all. Whilst other diseases in salmon can be considered more of an emergency, gill pathologies are a universal condition that can and do effect salmon and almost all cultivated fish species (Bruno et al., 2013). Gill disease is also one of the more complicated disease conditions to treat and prevent, because of difficulties in elucidating the true causative agents involved. A multitude of agents can impact the health and integrity of gills of farmed salmon, with both infectious and non-infectious causes, and varied severity of disease (Bruno et al., 2013; Rodger et al., 2010; Speare, 2007). Determining aetiology of gill disease can be confusing though, due to the shared pathobiology of observable response to different infections within gill tissue (Roberts, 2012), and frequent incidence of mixed infections (Herrero et al., 2018).

1.1.3 Advances in preventing disease

With appropriate farm planning, management and monitoring, the effects of pollution, genetic and nutrition-induced causes of gill pathology have been minimised in aquaculture (RSCPA, 2015; SEPA, 2011). A number of infectious diseases are now successfully controlled with biosecurity protocols and routine vaccination of fish, such as vibriosis in Atlantic salmon (Van Gelderen et al., 2009), although others are less well managed. Various technologies are employed in the attempt to control sea lice (*Caligus elongatus* and *Lepeophtheirus salmonis*), including use of cleaner fish, treatment of salmon with veterinary chemicals (Jackson et al., 2018; Pike et al., 1999) and hot or freshwater treatments (Overton et al., 2019) Cleaner fish used includes *Labrus bergylta* (ballan wrasse) and *Cyclopterus lumpus* (lumpfish), housed alongside farmed Atlantic salmon in sea cages for biological control of sea lice (Jackson et al., 2018).

The presence of non-infectious but harmful environmental organisms in the marine environment, such as phytoplankton and cnidarian jellyfish which appear capable of a serious impact on fish health, are particularly difficult to predict and mitigate. Exclusion of microscopic organisms from sea cages is not possible with the current net pen system of marine production. Incidence too of these organisms within the aquatic environment can rapidly change with environmental conditions for locally propagating blooms, or influx of large pelagic blooms into tidal locations by prevailing winds (Lynam et al., 2004; Purcell et al., 2007).

1.2 Gill structure and function

1.2.1 Gross structure

The structure of gills varies between species of fish. However, the general design and function remain the same. A large surface area assists in facilitation of gas exchange and osmoregulation, and protective mechanism reduce the risk of damage or disease. Salmonid gills are composed of four layers of branchial gill arches under the bony operculum that protects underlying gill tissue (Laurent, 1984). Each bony gill arch supports a double layer of comb-like gill filaments, which are long cartilaginous

extensions from the arch with a thin covering of epithelial tissue. These are arranged on top of each other to form the branchial arch gill fan (Bruno et al., 2013). In addition to the four distinct gill arches, a fifth, more vestigial area of gill tissue known as the pseudobranch is also present in the majority of salmonids, with an apparently more immune-associated function than respiratory (Laurent and Dunel-Erb, 1984). Each branchial arch contains many hundreds of individual gill filaments, and projections known as lamellae cover the surface of each filament. Lamellae extend perpendicular from the primary filaments to further increase the surface area of gill tissue (Figure 1.2).

Water passes over the gills by entering through the mouth and passes over the gills before being expelled via the operculum. Gas exchange then occurs through the squamous epithelial cells that cover the lamellae, which is only one to two cells thick over a basement membrane to facilitate diffusion. Gills are amongst the most vulnerable of anatomical features due to their external location and contact with water, as well as due to their delicate structure (Bone et al., 2008; Roberts, 2012).

Figure 1.2 Gill anatomy

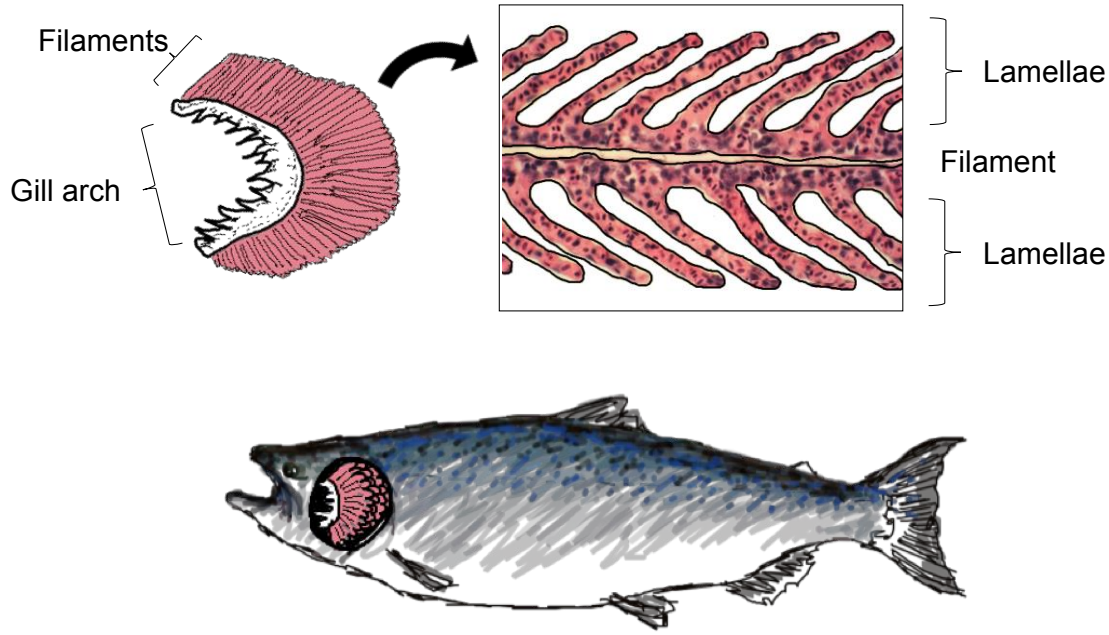


Figure 1.2*: Gross anatomy of gill structure in salmonids. Branchial arch and radiating filaments can be observed in-situ following removal of the operculum. The cartilaginous arch supports many hundreds of filaments. Filaments contain further cartilaginous support as well as an extensive network of vasculature, and a covering of mainly epithelial cells. The magnified view allows observation of a filament cross-section, with central venous sinus and secondary lamellar structures extending perpendicular to the filament visible*.

1.2.2 Microscopic structure

Much of the architecture of gill tissue, including sub-surface anatomy and individual cell types, cannot be observed through gross assessment alone. These structures can, however, be visualized using imaging technology, such as histological sectioning and microscopy (**Figure 1.3**). The microscopic structure of gills consists primarily of epithelial cells that cover 90% of the gill surface in teleost fish such as salmon (Evans et al., 2005). Surface epithelial cells are squamous (and known as pavement cells), above sublayers of largely undifferentiated cells and a basement membrane. Epithelial cells are arranged only one to two cells thick over a basement membrane on the lamellar surface, to facilitate gas exchange (Wilson and Laurent, 2002), whereas cell layers are

thicker over the remainder of filament tissue, such as at the base of lamellae and across the tissue of the gill arch (Bruno et al., 2013). Epithelial cells possess tiny microvilli projections on their environmental facing surfaces that contribute to increasing the gill surface area (Wilson and Laurent, 2002). The large surface area of gills facilitates gas exchange, with lamellar epithelial cells providing a thin layer through which oxygen and carbon dioxide (CO_2) can diffuse.

Besides pavement cells, various other cell types make up the ultrastructure of the gill surface, all with important roles in gill function. Pillar cells are modified epithelial cells that support the lamellae, providing structure and definition to capillary spaces through association with collagen (Bettex Galland and Hughes, 1973). Gill tissue has a high vascular demand and beneath the surface of each filament runs a central venous sinus from which capillaries extend into the lamellae (Al-Kadhomiy, 1984). This creates a dense network of blood vessels within gills. As well as movement of respiratory gases, the circulatory system can deliver cells with immunological function such as leukocytes in response to gill insult. Mitochondria-rich cells also known as ionocytes, perform an osmoregulatory function in gill tissue, facilitating ion exchange through various types of pump (Galvez et al., 2002). The chloride cell is a commonly observed ionocyte, important for osmoregulation through chloride ion exchange (Foskett and Scheffey, 1982). This function is essential particularly in anadromous fish such as salmon that transition between marine and freshwater environments, with increased chloride cell number and size reported in salmonid gills following the transition to saltwater (Zadunaisky, 1996). Cell function and uptake or excretion of charged particles is altered with osmotic pressure of the aquatic environment. Goblet cells are present within gill tissue for production of mucus. These are large, ovoid cells, containing mucus-secretory granules, most commonly observed on the surface of lamellar tissue. The number of these cells can also vary with different environmental stimuli and stressors (Karlsen et al., 2018; Padra et al., 2014). Mucus has important osmoregulatory and gas exchange function in gills. It also plays an important protective role, with physically protective and immunological properties (Gomez et al., 2013a; Ultsch and Gros, 1979). Mucus upon the gill surface forms a protective layer above the thin epithelium layers of delicate lamellae below (Lumsden et al., 1994), as well as containing many

immunological compounds (Koppang et al., 2015). These compounds protect the gills, as well as assisting in maintenance and modulation of the resident microbiota of the gills (Koppang et al., 2015; Lowrey et al., 2015).

Numerous immune cell types (leukocytes) can also be found within gill tissue, including eosinophilic granule cells, fixed macrophages, lymphocytes and rodlet cells (Bruno et al., 2013; Reite and Evensen, 2006; Roberts, 2012). These immune cells function in the defence of tissue, driving protective responses such as inflammation and adaptive immunity. Different cell types have different action in immune defence, such as phagocytosis of foreign material by macrophages, or degranulation of eosinophils as part of the response to parasites (Koppang et al., 2015). Both B and T-type lymphocytes are active within gill tissue for immunoglobulin production through both humoral (innate) and adaptive immune responses (Nakanishi et al., 2015; Salinas et al., 2011). A combination of these responses is important in the defence of gill tissue from the varied potential environmental insults, with non-specific humoral response to novel infectious agents and adaptive response as part of acquired immunity. Inflammation, with infiltration of immune-associated cells and altered genetic expression of gill tissue, is an important component of both. Leukocytes as part of the gill-associated lymphoid tissue (GIALT), are generally located within the deep tissue layers. GIALT is mainly located in the inter-branchial lymphoid tissue at the base of filaments, alongside neuroepithelial cells. Neuroepithelial cells in the gills function in oxygen sensing and circulatory control (Zachar and Jonz, 2012).

1.2.3 Function

Gills are required for osmoregulation and acid-base regulation, as well as oxygen uptake, carbon dioxide and nitrogen excretion and hormone metabolism, and they also function as sense organs. Without gills, fish could not obtain oxygen from the relatively poorly saturated aquatic environment (Maxime et al., 1991). Oxygen is essential for respiration and production of energy in the form of ATP through metabolic pathways, and the gills of salmonid fish are therefore structurally organized to facilitate rapid gas

exchange for efficient respiration. A balance between respiration and osmotic and physical protection must exist, however.

Respiratory exchange occurs by diffusion of oxygen into the vasculature across the epithelial layer of gills, and diffusion of carbon dioxide in the opposite direction. Respiration requires sufficient available dissolved oxygen, a good blood supply containing sufficient haemoglobin, and a large functional gill surface for exchange (Bone et al., 2008; Mallya, 2007). Any form of pathology of gill that alters tissue structure or occludes will impair gas exchange, potentially leading to anoxia. Impaired uptake of oxygen will have physiological and behavioural consequences such as 'gasping' behaviour or reduced activity to conserve energy and maintain cellular oxygen homeostasis (Kramer, 1987). Reduced activity can manifest as a lack of feeding activity, leading to poor growth and increased susceptibility to secondary pathogenic insult. Studies have shown a reduction in metabolic rate and food conversion in multiple species during anoxia (Mallya, 2007; Pichavant et al., 2001; Svobodova et al., 1993; Verheyen et al., 1994), as well as further physiological stress responses. Fish therefore have various behavioural and physiological mechanisms to optimize oxygen uptake as part of a stress response to anoxia, with systemic and cellular responses. Systemic adaptation includes altered hormonal and neurological signalling for physiological adaptation, including altered cardiovascular output (Gattuso et al., 2018).

Gills also have important function in osmoregulation. Euryhalinity is the ability to osmoregulate across a range of salinities, an ability possessed by members of the Salmonidae family. Salmon experience both hypo- and hyperosmolarity challenges during their lifecycle, and are specially adapted to cope (Bone et al., 2008). The saline marine environment, for example, exerts a hyper-osmotic stress, encouraging the loss of water from the gills and influx of ions. Passive ion loss and water entry occur in the freshwater environment, which salmonids counteract by active uptake of sodium and chloride across the gill. In seawater, dehydration and passive ion gain must be counteracted by expulsion of excess sodium and chloride by the gills (McCormick et al., 2009). Thus, the transition from freshwater to seawater requires that the gills reverse their function from an ion uptake to a salt secretory organ, through passive and active

action of ionocytes (Devlin et al., 2000; Evans, 2002; Handeland et al., 1998; Karnaky, 1986; Mccormick et al., 2013; Zadunaisky, 1996). Mucus plays an important role in the function of osmoregulation, through reduction of the osmotic stress of the external environment (Evans et al., 2005). Drinking too alters the physiological demands of fish, with osmoregulatory gut activity. Damage to the gills alters the ability of fish to osmoregulate, and negatively impacts survival (Sloman et al., 2006). As well as maintaining osmolarity (Devlin et al., 2000; Evans, 2002; Handeland et al., 1998; Karnaky, 1986; Mccormick et al., 2013; Zadunaisky, 1996), ionic regulation is also important in fish as part of the acid-base transfer for pH regulation (Claiborne et al., 2002). Respiratory adjustment of bicarbonate buffer system and transport of molecules between the fish and environment are essential gill-associated functions as part of acid-base regulation (Evans et al., 2005).

Figure 1.3 Microscopic anatomy of gills

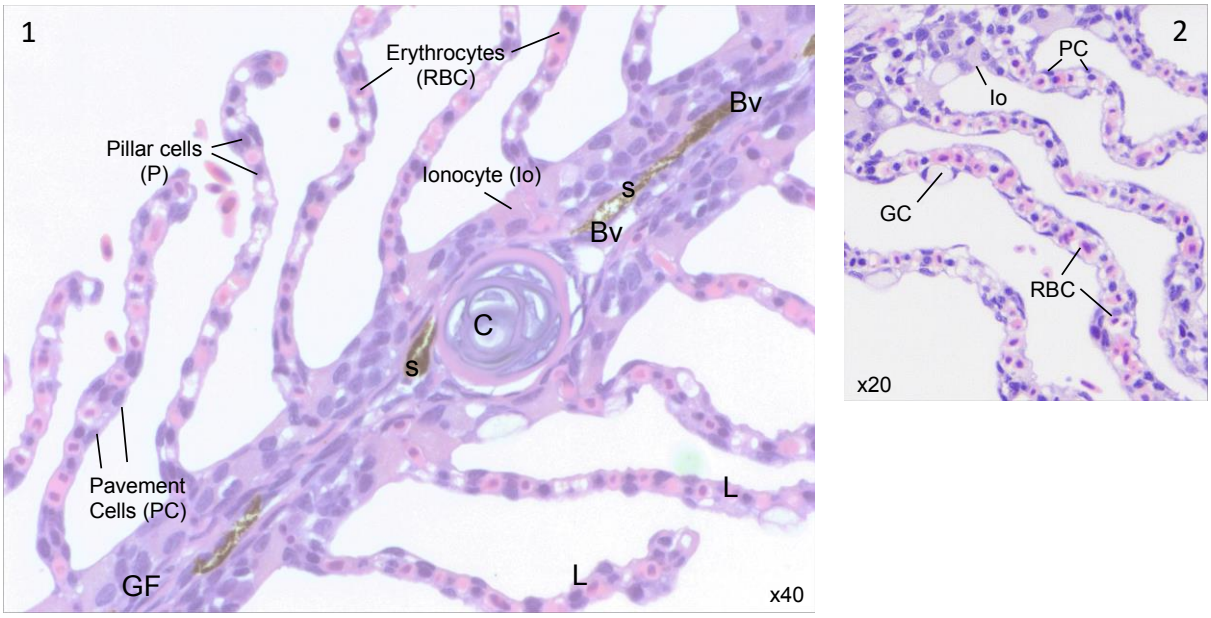


Figure 1.3*: Images illustrate the microscopic structure of healthy gill tissue at x40 (Image 1) and x20 (Image 2) magnification. Sagittal sections with H&E staining for observation of normal gill architecture. Squamous pavement (**PC**) and pillar (**P**) epithelial cells are highlighted giving structure to the lamellar (**L**) tissue. Erythrocytes (**RBC**s) are visible within the lamellar capillaries. Some RBCs are also present outside tissue as artefact resulting from sample collection. Osmoregulatory (**Io**) and goblet (**GC**) cells are visible within respiratory surface too. **GF**: Gill filament. **L**: Lamellae. **C**: Cartilage. **Bv**: Blood vessel. **s**: Artefactual staining.*

Gills are amongst the most vulnerable of anatomical features due to their external location and contact with water, as well as due to their delicate structure (Bone et al., 2008; Roberts, 2012). Direct contact with the surrounding environment means gills are constantly exposed to infectious and harmful agents. Gills are an externally facing mucosal membrane in fish, producing mucus and providing immunological protection of underlying tissue (Koppang et al., 2015). Unlike the externally facing human mucous membranes found lining respiratory, urogenital and digestive tracts, the epithelial covering of gills is thin, to facilitate its other functions. This makes gills particularly susceptible to trauma (Rodger et al., 2010). No keratinisation or scales are present to protect underlying tissue, making mucoid protection of gill tissue highly important. A mucus layer composed of polysaccharides and water is secreted by the mucosal surface to protect underling tissue from trauma. It contains too various bioactive molecules, including for example antimicrobial peptides, for prevention of bacterial overgrowth and ingress of microbes via the gill tissue (Koppang et al., 2015; Koshio et al., 2016; Lumsden et al., 1994). Mucus represents an essential innate defence against infection in fish, as well as performing additional important functions in chemical communication, and hosting components of the resident microbiota. Mucus composition of gills varies from that of the skin, suggesting a specificity of function across these two layers in salmonids (Lumsden et al., 1994; Lumsden and Ferguson, 1994). Gills therefore represent an important immunological organ in fish, with the gill tissue demonstrated to express transcripts for most immune-related genes identified in teleosts (Koppang et al., 2015). The lymphoid GIALT, immune cells dispersed through the tissue and even epithelial cells themselves can all express genes with immune function (Dalum et al., 2015)

1.2.4 Microbiome

In addition to the cells and tissue structures of fish themselves, the microbial communities of mucosal surfaces also play an important role in the function of fish tissue. The term microbiome describes the collective community of all the microorganisms living in and on a tissue surface, including bacteria, archaea, viruses, and fungi (Foster et al., 2018; Mcfall-ngai et al., 2013). The bacterial consortia are of

particular interest to modern research, as they are proposed to contribute in multiple ways to the survival of their host, such as in defence, and nutrition (Beck et al., 2015; Flórez et al., 2015; Nicholson et al., 2012). In return, these microbes can proliferate in a nutrient rich, relatively uncompetitive environment. Whilst these bacteria are presumably acquired from the environment, the composition of the microbiota is often quite different from that of surrounding free-living bacterial communities (Ley et al., 2008). The adherent bacteria are thought to exist in a dynamic population, with composition controlled by the host at least to some extent (Ikeda-Ohtsubo et al., 2018; Maynard et al., 2012).

A host and its adapted microbial communities can be known collectively as the holobiont (Pita et al., 2018; Shapira, 2016; Zilber-Rosenberg and Rosenberg, 2008). Pathogens have long been considered drivers of natural selection (Haldane, 1932), but recent research suggests the impact not only of harmful bacteria on host evolution, but also commensal and symbiotic microbiota. In addition to their real-time assistance in success and survival of their hosts, it is possible mutualist microbes might act as facilitators of niche adaptation and speciation of hosts over time (Brucker and Bordenstein, 2012). The hologenome hypothesis proposes that association between a host and its symbionts affects the fitness of both (Moran and Sloan, 2015; Zilber-Rosenberg and Rosenberg, 2008). Exchange in microbes (and their genes and associated functions) with the environment can facilitate host adaptation (Shapira, 2016), not to mention the potential for direct exchange of genetic material between microbes and their hosts.

1.2.4.1 Function of the microbiome

Host tissue is thought to provide a nutrient-rich environment for microbial growth, and previous research has suggested a symbiotic relationship of microbes and vertebrate hosts that traces back over millions of years (Brugman et al., 2018). The theory of a core gut microbiome containing symbionts is being extensively explored in humans and many authors believe the same concept might exist in bony fish. Microbiota are proposed to be involved in a number of body processes and functions important to host

survival in humans. Potential interactions and impacts on host functions include involvement in metabolism, immunity, development, fecundity and even behaviour (Sampson and Mazmanian, 2015; Sison-Mangus et al., 2015).

The microbiota of the GIT in particular is extensively studied in all animals, and a focus of research for the study of fish utilised in aquaculture. Studies in fish indicate that the bacterial assemblages of the hindgut in particular are clearly divergent from environmental populations (Salinas et al., 2011), with autochthonous communities of microbiota more closely mimicking that of mammalian digestive assemblages (Sullam et al., 2009). Collectively, the published material indicates that fish harbour specialised microbial communities. Whilst a limited number of examples of true symbiosis have so far been published (Clements and Bullivant, 1991; Fishelson et al., 1985), a clear association with diet has been established (Beck et al., 2015; Ley et al., 2008; Zha et al., 2018). The function of these microbes are clearest in certain herbivorous fish such as *Crinodus lophodont* (sea carp) that rely on the activity of gut microbes to convert unassimilable algal molecules to short-chain fatty acids (Seeto et al., 1996). True hindgut fermenting fish therefore exist in a symbiotic relationship with microbiota of which that aid in assimilation of digesta resistant to host digestive enzymes (Clements et al., 2014; Mackie, 1997; Mountfort et al., 2013).

The function of GIT microbial populations in omnivorous fish such as salmonids is less clear. Many physiological functions have been proposed based on known properties of microbes identified using high-throughput sequencing or gene expression studies. Many of these proposed mutualistic relationships remain however to be fully characterised, including for example microbial activity in degradation of digesta through complimentary pathways of metabolism, production of bioactive compounds or a role in detoxification (Clements et al., 2014; Egerton et al., 2018). Studies in zebrafish though do demonstrate a role for gastrointestinal microbes in stimulating fatty acid uptake (Carmondy and Turnbaugh, 2012). Research has demonstrated the ability of gut-derived microbiota in fish to produce compounds associated with the stimulation of gut motility (Rawls et al., 2007) and development (Bates et al., 2006) in zebrafish, suggestive of wide-ranging essential functionality. Microbes of the gastric tract have even been

demonstrated in other animals to have activity in appetite regulation and metabolism (Duca et al., 2012). The research topic of gut microbiota is subject to much inquiry as greater knowledge of nutrient uptake and metabolism in fish might enhance their aquaculture production.

The immunological properties of microbial communities are similarly of interest to those concerned with the health and disease-resistance of aquacultured fish. It has been proposed that the gastric microbiota of salmonids might function too as a defensive barrier against pathogenic taxa, through assisting in maintenance of the integrity of the epithelial surface (Ringø et al., 2007, 2003, 1995). Zebrafish models demonstrate the requirement for commensal microorganisms in development and function of the immune system both during homeostasis and with disease (Murdoch and Rawls, 2019), and resident bacteria of the GIT that produce compounds that can function in community remediation have been isolated from the number of fish species. In humans the microbiome has even been linked to modification of behaviour; cognitive function, social interaction and even stress management all appear to be impacted by microbial populations through the proposed action of neuroactive compounds (Dinan et al., 2015). It is possible that the enteric microbiota of fish might have similar far-reaching consequences for health of individuals.

In addition to exploration of the microbial communities of digestive compartments, recent research has focused too on the microbial communities of other important defensive mucosal surfaces, including skin and gills (Arias et al., 2019; Legrand et al., 2018; Lowrey et al., 2015). Existing research has identified bacteria with a potential role in ammonia cycling in gill tissue(Van Kessel et al., 2016), as well as microbes with the capacity for community modulation and production of antimicrobial peptides (Pratte et al., 2018).A function of particular interest in the aquaculture production of salmonids would be the ability of microbiota to assist in the immune and stress response of their host to negative stimuli, such as disease. Commensal microbes within adherent biofilms are known to compete with others for resources, and even produce compounds such as anti-microbial peptides that can impair the growth of microbial competitors (Gomez et al., 2013b; Wanka et al., 2018). This might act too in the interest too of their host,

through prevention of overgrowth of any single bacterial population, and inhibition of the activity of bacterial pathogens (Kamada et al., 2013). The microbiota might then assist in the host response to stress through production of protective compounds during xenobiotic exposure that limit damage to host tissue (Carmody and Turnbaugh, 2014) or even confer direct pathogen resistance to their hosts through specific priming of the host immune system (Montalvo-Katz et al., 2013).

Commonly cited symbiotic relationships of aquatic organisms include that of coral and nitrogen fixing bacteria (Lema et al., 2012), and pufferfish and its microbial symbionts in toxin production (Simidu et al., 1990). Based on the existing information in mammals and the expanding knowledge of fish, there certainly seems to exist the potential for varied involvement of the microbiome in the biological functions of fish. Although much is still to be learnt regarding the full spectrum of involvement in fish host function and health that microbiota can have, particularly for gill tissue, the microbiome might well represent an important factor in and indicator of fish health.

1.2.4.2 Establishment and maintenance of the microbiome

Young fish

All aquatic species host adherent microbiota, and all appear to be distinct from environmental populations and specialised in some way. Recent research in fish has focused particularly on the microbiota of the digestive tract, where even closely related species appear to have microbial communities distinct from one another (Reverter et al., 2017). Microbiota colonising the mucosal surfaces of fish do so because of a complex combination of factors. Whilst mammals are thought to obtain their initial microbial bolus from their mothers, with this initial transfer impacting community composition and even new-born survival (Schokker et al., 2014; Vallès et al., 2014), colonisation with bacteria of the mucosal surfaces of fish appears to be predominantly horizontal transfer from environmental populations (Ikeda-Ohtsubo et al., 2018; Sylvain and Derome, 2017; Webster et al., 2018). Even before hatching, a microbiome is present on the surface of eggs, with bacterial components therein such as Actinobacterial taxa

demonstrated to impact the success of disease-causing organisms, through inhibiting attachment of the fungal pathogen Saprolegnia (Liu et al., 2014). Any immunologically advantageous function of these bacteria would be particularly important in juvenile fish due to the immature status of their immune system development (Liu et al., 2014). Colonisation of mucosal surfaces of freshly hatched alevin occurs after they emerge from eggs (Brugman et al., 2018). The bacteria that then form the adherent communities of tissues are likely recruited from environmental populations (Bright and Bulgheresi, 2010), and therefore the rearing conditions of emergent fish are crucial, with the potential to impact all subsequent life stages within a system of production (Dehler et al., 2017a; Vdastein et al., 2018). Anexic (microbe-free) zebrafish, for example, fail to develop properly their gastrointestinal tract (GIT) (Bates et al., 2006), demonstrating reduced levels of enteroendocrine and goblet cells, a lack of brush-border activity in detoxification, and a loss of epidermal integrity (Bates et al., 2006; Rawls et al., 2007).Whilst there is likely limited transfer of mutualistic microbiota from parent to offspring in fish relative to the vertical transmission that occurs in mammalian hosts, coevolution of organisms has still been proposed in aquatic species (Boutin et al., 2014; Vega, 2019), with proposed adaptation of a consistent or 'core' microbiome to fish mucosal environments Initial colonising microbial communities demonstrate low diversity, with dominant populations of a small number of phyla increase in diversity over time (Lee et al., 2000; Vega, 2019). However, in the microbiota of Atlantic salmon, wild counterparts are consistently identified as more diverse populations than aquaculture-reared equivalents. Microbiota contained therein are similar but not identical (MacFarlane et al., 1986; Roeselers et al., 2011). The cause of this divergence is likely due to a confluence of factors, including diet, environmental conditions and host genetics, however This variation in microbiota might though be important in the functionality of their microbiomes.

The various mucosal organs and epithelial surfaces in young fish, including the skin, gills and digestive tract, are rapidly colonised by microbes to establish a resident population. However, not all environmental bacteria are incorporated into the microbial populations of these mucosal organs. Although the microbiota of new-born animals often resembles their environment (Dominguez-Bello et al., 2010), acquisition and

structure of the resident microbiota appears to be rapidly modified, with microbial succession proposed to eventually establish a stable community of bacteria (Vallès et al., 2014), reflective of the host environment (Ikeda-Ohtsubo et al., 2018) but also specific to host species (Larsen et al., 2013).

Compartment specific communities

Microbial community composition varies both with environmental conditions and host factors. Discrete communities of microbes can establish in different tissues, with significant differences noted between the communities of gills and skin (Legrand et al., 2018; Webster et al., 2018), proximal and distal GIT (Moran et al., 2005) and even different populations between the lumen, mucous, brush border mucosa and intestinal crypts of the same region of fish intestine (Lyons et al., 2015). These therefore represent niche compartments, with distinct microbiota. Resident and non-pathogenic microbes can even exist intracellularly, as reported in the colonisation of intestinal epithelial cells (Chow and Mazmanian, 2011).

Host factors

Physical constraints of the host and tissue environment exert a direct impact on acquisition of bacteria, including through tissue environment and production of immunomodulatory compounds for the control and manipulation of microbiota (Dehler et al., 2017a; Gomez and Balcazar, 2008; Llewellyn et al., 2014). The viscous nature of mucus binds microbes, assisting in acquisition of bacterial populations, however the immune function of gills appears to act in a selective manner, suggested to target disease-causing or otherwise undesirable microbes and not the useful (or at least not harmful) adherent microbes (Merrifield and Rodiles, 2015). As mentioned previously, mucus contains a number of bioactive compounds with immunological function, as well as acting as physical protection and acting as part of physiological responses to stimuli such as osmotic stress (Evans and Somero, 2008). Selective pressure exerted by the host through tissue environments and immunological control is therefore considered to

modulate the microbiome and can restrict growth of undesirable microbes (Legrand et al., 2018; Ley et al., 2008; Llewellyn et al., 2014).

Host genetics are proposed also to play a role in microbial recruitment in salmonids (Brown et al., 2019). Mammalian research in the interaction of host and associated microbial communities identified genetic factors in humans that impact bacterial community composition, apparently through their enhancement or failure to promote a stable microbial community (Goodrich et al., 2017). Genetics, particularly elements with involvement in immune function and gastric architecture (Hall et al., 2017), have a proposed role in shaping the recruitment of microbiota, with potential implications in a number of human disease conditions, including Crohn's, Irritable Bowel Syndrome (IBS) and various dermatological disorders (Gevers et al., 2017; Menees and Chey, 2018; Petersen et al., 2018; Williams et al., 2019).

*Environmental factor*s

In addition to host factors, external environmental conditions and availability appear to have important influences on microbial community structure. Fish are exposed to a plethora of bacterial organisms in the aquatic environment, and this environment appears to play a key role in the establishment and maintenance of fish microbial populations. Besides the apparently crucial initial recruitment of core microbial populations from the environment, failure of which is proposed for farmed fish reared in artificial environments (Bates et al., 2006; Burns et al., 2017; Dehler et al., 2017a; Wong et al., 2013), continued exposure to environmental bacteria also impacts community structure. Altered environmental conditions introduce new microbes to mucosal surfaces, allowing for and potentially necessitating adaptation of community structure (Ornelas-García et al., 2018). Altered external conditions such as temperature and pH have been demonstrated to prompt altered taxonomic structure in the malleable microbiota too (Kokou et al., 2018; Sylvain et al., 2016). This might represent microbial adaptation, but also impaired survival of advantageous or at least benign residents, fora negative restructuring of microbial communities. Dietary composition clearly plays an important impact in microbial recruitment and structure of the GIT (Dehler et al.,

2017b; Michl et al., 2019; Vdastein et al., 2018), however it has also been demonstrated to impact the mucosal surfaces of other organs too, and overall health of fish (Legrand et al., 2018). Gastrointestinal communities have been observed to vary with different nutritional requirements of host organisms at different salmonid life stages as well as with altered diet (Kurokawa et al., 2007; Yatsunenko et al., 2012). Environmental factors specific to aquaculture appear definitively to impact microbial communities, likely through a combination of factors; Stress can lead to alteration of microbial populations (Zha et al., 2018), and stocking density appears influential also (Romero et al., 2014).

A further consideration in anadromous fish such as salmon is adaptation to different salinities, as fish move between seawater and freshwater environments during the course of their life cycle. Transition from fresh to salt water environments requires physiological adaptation, and with this comes adaptation too of the microbial communities (Lokesh and Kiron, 2015). Marine and freshwater environments can host highly dissimilar communities of microbes that might be adopted into the microbiomes of fish (Logares et al., 2009; Methe et al., 1998), and exert different infectious challenges (Belkin et al., 2005; Bowman et al., 2004; Mitchell et al., 2011a; Safińska, 2018). The transition from fresh to marine environments of salmonids appears an important driving factor in microbial community remodelling of skin and gill surfaces (Lokesh and Kirin, 2016). What constitutes the 'core' microbiota varies between species, however it is clear from the distinct and specialised communities of salmonid tissue that selective recruitment of microbes is occurring (Legrand et al., 2018; Reverter et al., 2017).

1.2.4.3 Core and transient microbial populations

Compositional similarities even across species hint at optimal microbiota depending on the functional requirements of the host. The rumen of bovids, for example, hosts a mixture of archaea and eukaryotic microorganisms as well as bacteria that assist in digestion (Wang and Mcallister, 2002), as do the digestive tracts of other vertebrates. Protobacteria is consistently identified as a dominant phylum in studies of fish, other

animals and plants (Ikeda-Ohtsubo et al., 2018), with a proposed function in assisting the breakdown of organic compounds, to facilitate change in the organochemical conditions of their environment (Bennett et al., 2009). The specifics of community composition do however appear to vary between organisms. Enterobacteriaceae dominate the proteobacterial community in terrestrial livestock (Mao et al., 2015; Stanley et al., 2014), whereas the gammaproteobacterial microbes Aeromonadaceae and Vibrionaceae appear to characterise the intestinal microbiota of freshwater and saltwater fish respectively (Sullam et al., 2009; Tarnecki et al., 2017). Existing research supports the existence of 'core', tissue specific, microbial communities within host species (Roeselers et al., 2011; Shapira, 2016; Webster et al., 2018). Microbes identified a part of a 'core' population are considered to be microbes present across populations and varied environmental conditions that might be determined to be well adapted symbionts (Berg et al., 2016; Roeselers et al., 2011), however true symbiosis is yet to be demonstrated in salmonids. It is theorised though that these organisms have a mutualistic relationship with their host, where microbes perform essential functions in their particular system or partition (Ikeda-Ohtsubo et al., 2018). Different 'core' microbiota observed in different host species are thought to be due to varied functional requirements (Reverter et al., 2017).

Significant interindividual variation in microbiota composition is however also often identified (Boutin et al., 2014; Fjellheim et al., 2012; Webster et al., 2018). It appears therefore that any core microbiome is supplemented by additional microbes obtained from the environment, a pool that varies with environmental conditions. Whilst 'core' microbiota are suggested to remain constant, variable communities of apparently transient facultative bacteria are thought to be the drivers of intraspecific variation (Nayak, 2010). These microbes are those thought to vary with available environmental diversity, individual host and external factors such as disease or nutrition. This allochthonous pool contains a mixture of commensal microbes and opportunistic pathogens, both of which have been observed in adherent bacterial communities of a variety of fish species. The presence of these microbes might allow for more varied adaptation, however the ability of the microbiome to host varied microbial populations comes with the associated risk of negative impact too. Controlled alteration of the

microbiota with prevailing environmental conditions is thought to improve host survival, assist in adaptation and even confer disease resistance (Dhanasiri et al., 2011; Gao et al., 2013; Kokou et al., 2018). However, recruitment of inappropriate microbes might lead to disease. Animals and plants therefore appear to harbour complex and diverse microbial communities constituting a mixture of well adapted and more generalist, or transient, populations too. Current thinking is that microbial assemblages of fish tissue are composed of a mixture of taxa, including microbes that might be considered symbionts, commensals, and even opportunistic pathogens (Brugman et al., 2018; Gobet et al., 2018; Roeselers et al., 2011)

1.3 Complex interactions and causes of gill pathology

Despite protective and compensatory mechanisms within gills, a multitude of factors have been observed to negatively impact gill health. Protective mechanisms can be overwhelmed or disrupted, leading to pathological change in gill tissue (Bruno et al., 2013). Both infectious and non-infectious stimuli can illicit pathological change in gill tissue via varied mechanisms of gill trauma and disease.

1.3.1 Infectious agents of gill disease

Pathogenic viruses, bacterial prokaryotes and eukaryotes including parasites, protozoans and fungi are all infectious agents of gill disease in fish. Depending on host fish species and the environment in which it lives, specific pathogenic organisms can vary, but there exist hundreds of recognized pathogens of the many species of fish, with more described every year. Salmon Pox Virus, for example, is a recently described gill pathogen in salmonids, which is described as an emerging pathogen, but in fact is one that likely went undetected for an extended period of time before isolation as the aetiological agent of gill disease (Gjessing et al., 2015; Kvellestad et al., 2003; Nylund et al., 2008). Other infectious gill conditions in salmonids are still not fully described, including epitheliocystis, a disease with multiple proposed causative agents, including *Candidatus Branchiomonas, Candidatus Piscichlamydia* and *Candidatus Fritzia* (Steinum et al., 2010; Toenshoff et al., 2012). Better described bacterial diseases of

salmonids include Tenacibaculosis, caused by infection of gills with *Tenacibaculum maritimum*, as well as other generalised epithelial infections such as Vibriosis and Furunculosis, caused by *Vibrio* sp. and *Aeromonas salmonicida* respectively (Belkin et al., 2005; Lafferty et al., 2015; Meyers et al., 2008; Mitchell et al., 2011a). Sea louse copepods traumatise fish through attachment and grazing on mucus, blood, and epithelial tissue (Bruno et al., 2013) and are second only to the purchase of feed as the largest financial outlay for the Atlantic salmon industry (Costello, 2009). A further emerging disease in production of Atlantic salmon globally is the gill disease termed Amoebic Gill Disease (AGD), an infection associated with severe gill pathology. AGD is a major cause of fish mortalities in marine production of Atlantic salmon (Mitchell et al., 2011a; Rodger and Mcardle, 1996). This disease appears to be caused by a protozoan amoeba; *Neoparamoeba perurans* is reported as the principal causative agent (Crosbie et al., 2012), although other amoeba have also been associated with the disease in salmonids (Bermingham and Mulcahy, 2007). Fungal infections are also problematic in Atlantic Salmon, however, these generally occur in the freshwater environment (Bruno et al., 2013).

1.3.2 Non-infectious agents of gill disease

Gill disease can be initiated and exacerbated by many non-infectious and even non-biological causes. Extensive literature exists regarding the impact on fish from organic pollutants, heavy metals and other chemicals that might enter the water system as pollution (Sindilariu et al., 2009; Zeitoun et al., 2014). Altered gill structure with impaired function has also been observed in aquaculture-reared fish following relatively routine on-farm activities. This includes handling, and exposure to hydrogen peroxide (H_2O_2), utilised in many salmonid hatcheries and for the treatment of sea lice in Atlantic salmon (Kiemer and Black, 1997; Ort et al., 2002; Palomba et al., 1999).

Of increasing concern to the aquaculture industry, at least in production of Atlantic salmon, are cnidarian zooplankton and phytoplankton (algae) (Rodger et al., 2010). Little research exists regarding these harmful environmental organisms and their impact on gill health, but what there is suggests the potential for significant negative impact on

fish productivity and survival. Although the acute impact of large aggregations of cnidarian organisms (commonly known as jellyfish blooms) is well understood to lead to mass fish mortalities, research has only recently begun to address the questions of the mechanisms of negative impact, and consequence of lower intensity exposure.

The impact of phytoplankton on fish health is relatively better understood. Harmful Algal Bloom (HAB) forming phytoplankton are known to harm aquatic organisms and even terrestrial animals through their toxic products; bioaccumulation in the food-chain can lead directly to poisoning of higher organisms that ingest toxic material (Berdalet et al., 2015). Phytoplankton of public health concern cause syndromes such as Neurotoxic Shellfish Poisoning (NSP) and Paralytic Shellfish Poisoning (PSP) in humans (Hinder et al., 2011; Springer and Holley, 2013). HAB-forming phytoplankton are, however, more varied than just those associated with toxic effects in humans. Some produce products that are directly toxic to aquatic organisms only, including the phytoplankton *Prymesium parvum* (Watson, 2001). Other harmful impacts of phytoplankton on fish health include alterations to dissolved oxygen concentrations (Rodger et al., 2010), and some algal organisms with silicate cell structures or sharp protrusions have been demonstrated to directly traumatise gill tissue (Reis Costa, 2014; The Scottish Government, 2006; Yang and Albright, 1992). Although better data exists regarding the impact of HAB-forming phytoplankton on different aquatic organisms, the variety and complex components of all toxic products are yet to be fully explored. Current research has focused on the constituents and impact of toxins of concern to human health, particularly in shellfish, due to their method of filter-feeding and association with poisoning cases.

1.3.3 Complex disease syndromes

It is apparent that numerous factors can illicit gill damage and pathology in fish. The intensive production system of Atlantic salmon aquaculture appears to particularly predispose these fish to gill diseases, however diagnosis remains problematic (Herrero et al., 2018). Microscopic analysis rarely results in diagnosis of a single causative agent, and exposure of gills to the environment can result in incidental findings of many

organisms, not only those causative of any observed pathology. Whilst laboratory challenge trials for investigation of individual infectious agents of gill disease provide important data regarding the action of pathogens, these are not representative of naturally occurring, often mixed infections. Most of the observed gill disease in farmed Atlantic salmon appears to be multifactorial in its cause, perhaps with initial trauma or infection predisposing to subsequent infections and pathology or a confluence of factors leading to overwhelming of the gill defences. With increasingly sophisticated diagnostic intervention, a combination of aetiological agents are frequently isolated from farmed fish within the marine environment (Herrero et al., 2018; Mitchell et al., 2011a).

Much previous research has studied gill disease in farmed fish, but much is still to be ascertained about the interaction and predisposing factors of the mixed infections observed in the marine environment. It has been suggested, for example, that bacterial agents of disease in gills are less frequently primary pathogens, and more often secondary opportunists, capitalising on impaired immunity or tissue disarrangement from existing conditions (Horn, 2008). The question of predisposing factors for gill disease is therefore an important field of study, with publications investigating the role of gill surface microbiota (Embar-Gopinath, 2006; Embar-Gopinath et al., 2005) and trauma from the cnidarian hydroid *Ectopleura larynx* that grows on sea cages (Bloecher et al., 2018) in initiation of AGD.

Descriptive terms such as Proliferative Gill Inflammation (PGI) Proliferative Gill Disease (PGD) and Complex Gill Disease (CGD) have been used to describe unknown or mixed aetiology disease (Herrero et al., 2018; Nylund et al., 2008; Steinum et al., 2010). PGI and PGD have been associated with salmon pox virus and cyst-causing bacteria from the phylum *Chlamydia* (Bruno et al., 2013; Nylund et al., 2008), with coinfection of Atlantic salmon paramyxovirus (ASPV) sometimes identified also (Kvellestad et al., 2005). CGD appears linked with *Desmozoon lepeophtherii* and *Candidatus Branchiomonas cysticola* infections (Herrero et al., 2018). Individually, many of these infectious agents can be isolated from fish gills demonstrating no apparent ill effects from their presence (Downes et al., 2015; Nowak and LaPatra, 2006; Orrego, 2015). *Candidatus Branchiomonas* for example has been identified as anormal

component of the gill microbiota of rainbow trout gills, observed without detectable tissue pathology (Brown et al., 2019). Clearly, therefore, these syndromes are multifactorial conditions with complex aetiologies. The involvement of predisposing factors for disease is not yet fully understood, and there may be further interactions of undetected pathogenic organisms associated with the pathology of disease. The 'epidemiological triad' describes the important interaction between host, infection and environment that influence the outcome and propagation of disease (Szklo et al., 2018), highlighting the influence of these factors in disease outcomes. The interaction of underlying concomitant infections of gill disease is therefore a key topic of inquiry for the industry and academic research. These broad disease terms provide a convenient catch-all for describing observed gill pathology, but they fail to inform regarding the disease processes at hand. Much research is still required before an understanding of the complex interactions and pathogenesis of these disease syndromes are achieved. What is apparent is that most gill infections appear multifactorial, with temporal changes in observation, likely due both to altered infectious challenge and host susceptibility.

Bacterial infections are many and varied, and are often thought to be opportunistic colonisations following impairment of the salmon defences (Belkin et al., 2005; Mitchell et al., 2011a). Infection can occur secondary to direct gill trauma, such as from physical damage or water-borne irritants such as jellyfish. Directly infectious agents in the marine environment include flexibacteriosis (also known as Tenacibaculosis) and furunculosis, both bacterial diseases affecting skin and gill epithelium (Avendaño-Herrera et al., 2006c; Bruno et al., 2013). The causative agents of these infections are documented as surviving poorly in the marine environment (Austin et al., 2012; Avendaño-Herrera et al., 2006c, 2006a), so it has been suggested that vector transmission might be involved in their spread. Previous research has explored the potential for jellyfish to act as transmission vectors for the causative agent of flexibacteriosis, *T. maritimum* (H. W. Ferguson et al., 2010), potentially predisposing fish to infection with the traumatic lesions caused by their stings. Microbial disease can certainly be transmitted by other aquatic organisms, with sea lice parasites implicated in the transmission of bacterial disease (Barker et al., 2009; Smage et al., 2016).

It seems reasonable to conclude that there is still much work to be done regarding gill disease in Atlantic salmon. The general conditions of complex aetiologies, with multiple factors and pathogens potentially contributing to the manifestation of disease, are particularly poorly understood. What unknown factors might yet be contributing further to the progression or prevention of these diseases? Sub-clinical gill pathology often goes undiagnosed (Rodger, 2007), but can be reasonably assumed to be having low-level effects on fish health and productivity through impairment of important gill functions. When no infectious agent can be isolated in association with observed pathology, disease is often attributed to 'waterborne irritants', a vague category that might include phytoplankton and cnidarian jellyfish. With their documented association with gill pathology, planktic organisms in the pelagic environment may well be the cause of unexplained or apparently complex gill pathologies observed in farmed fish. Research is, however, generally lacking as to the impact of these organisms, particularly at a sub-acute or sub-clinical level in fish.

1.4 Response to disease: manifestation and detection of gill pathology

Fish respond to challenges in a number of ways, with many methodologies utilised for detecting altered health status or impact of a stressor. Altered innervation and circulating hormones such as cortisol facilitate systemic response, including physiological mechanisms for altered homeostasis and alarm responses (Nilsen et al., 2015; Olsen et al., 2002; Roberts, 2012). Tissue-specific change can be observed through altered genetic transcription, utilized to infer mechanisms of response to specific stressors or traumas, such as in altered immune response (Chattopadhyay et al., 2014; Gattuso et al., 2018; Valdenegro-Vega et al., 2014). Epithelial and other cells are mediators of a variety of tissue responses to external stimuli to allow adaptation and survival of fish in a variety of conditions. Failure of compensatory mechanisms can also be monitored through gene expression for assessment of negative consequences in fish. Morphological change to the gill structure is observable with exposure to multiple stressors and altered gill structure can be reasonably utilized to infer functional impairment of gill tissue. Pathology can manifest in several ways, depending on the action of infectious agents and severity of tissue disarrangement. Infectious diseases

induce both shared and unique changes within gill tissue from their specific mechanisms of action.

1.4.1 Transcriptomic change

Even in fish maintained within a laboratory environment, free from pathogenic challenge, genetic expression occurs in all tissues as part of the maintenance of homeostasis and continued survival of the individual. Cell cycles are ongoing, with replication and eventual death, producing the products required for day to say survival and prosperity of fish. In the gills, genetic expression in cells facilitates the normal function of tissue, including for example the production of immune factors for maintenance of the microbiome, as well as production of mucus with important gas exchange and protective functions (Evans et al., 2005). During times of stress or traumatic insult, cellular response can alter the expression of key genetic elements for alteration of the gill microenvironment. Through altered transcription of genes, the functional response of tissue can be modified by all organisms to compensate for and response to change.

Transcriptomics is the study of RNA transcripts that are expressed from DNA, often studied through contrast of expression under specific circumstances relative to a 'normal' untreated control. In the case of gill cell culture, specific cell type responses can be assessed, while *in vivo* response studies generally assess the overall response of mixed cell-type tissues. The study of gene expression responses in salmonids has been used to study the tissue response of many organs to infectious challenge, as well as non-infectious harmful agents like heavy metals or chemical pollutants (Castro et al., 2016; Garcia-Reyero et al., 2015; Nam et al., 2018; Pillai et al., 2014). Transcriptomics also provides essential information regarding the transcripts associated with physiological adaptation in fish; studying the environmental stressors that induce altered gene expression for maintenance of homeostasis in fish provides insight into the control mechanisms of this adaptation.

Previous studies have demonstrated an array of transcriptomic changes in gill tissue to various stressors. Altered oxygen saturation, for example, induces change in the expression of *Hypoxia-inducible factor 1-alpha* (*HIF-a*) pathway genes, for downstream adaptive effects such as stimulating glycolysis, angiogenesis and erythropoiesis (Li et al., 2017; Nikinmaa and Rees, 2005). Certain stress situations such as parasite infection or osmotic challenge can also induce increased secretion of mucus (Speare and Ferguson, 2006). As well as acting in physiological adaptation, epithelial cells act as mediators of the immune response of gill tissue through expression of a variety of immune-associated transcripts. Whilst immune cell infiltration is certainly an element of the tissue response (Rieger et al., 2013), altered expression of the gill tissue itself is important too, particularly for modulation of commensal microbes on the gill surface that are part of the adherent microbiome. Inflammation-associated transcripts are observed as part of the general stress response, where as a distinctive pattern of response(with varied altered gene expression) can be induced in response to different kinds of infections, or other environmental insults (Castro et al., 2015). Epithelial cells can express various mediators of immunity, such as cytokines and antimicrobial peptides, which can be passed into the extracellular environment to form part of the mucus matrix. Leukocytes within the gill tissue, including those as part of the GIALT, also act in both the innate and immune transcriptomic response of gill tissue to stressors (Castro and Tafalla, 2015a). Environmental insults such as pollutants can induce antioxidant response pathways, and sometimes appear to impair immune function in some aquatic organisms (Gagnaire et al., 2004)

The expression of genes and pathways considered as part of the response to harmful stimuli or stress do not act exclusively in response to direct infection. Many stressors can induce an inflammatory response, including heavy metal toxicity, fertiliser run-off and even altered water temperature (Kostić et al., 2017; Lund et al., 2017; Morrison et al., 2006). Varied patterns of transcriptomic change are observed depending on the type of insult, with different responses observed for specific infective agents (Nam et al., 2018) and at varied level of insult (Martin and Dehler, 2016). These transcriptomic alterations can be utilised by researchers to gain an understanding of the function of genes, and the compensatory responses of tissue to varied insult. This has assisted in

understanding of immune response mechanisms in particular, with patterns of response also key to understanding the mechanisms of trauma. With AGD for example, study of the transcriptome has revealed genetic factors important in the pathogenesis of the disease (Morrison et al., 2006) as well as host resistance to infection (Boison et al., 2019). Many host-specific factors impact the response of individual fish, such as health status and age (Kijima et al., 2018)as well as taxonomic identity of fish (Gomes et al., 2019; Maekawa et al., 2019). Teleosts are reported as having a well-developed immune system similar in many ways to that of terrestrial vertebrates, (Castro and Tafalla, 2015b; Esteban, 2012), with a greater variety of available responses particularly compared to the more primitive immune repertoire of fish such as hagfish and lampreys (Buchmann, 2014). Study of the response of fish to stressors assists in the ongoing exploration of the similarities and variations within fish immune systems, for an understanding of their evolution and functional repertoire of immune function. Often the immune response appears at least partially contributory to the clinical effects of infectious disease, with negative consequences of response impairing tissue function (Škugor et al., 2009). A final important consideration is impairment of tissue function by disease or other stressors. Stress has been identified as an important cause of altered tissue function in salmonids, with associated impairment of immune function (Uren Webster et al., 2018). Damage to tissue structure will also result in loss of functional response.

1.4.1.1 Investigating transcriptomic change

The optimal transcriptional response depends on the characteristics of an infectious agent, and the biological action of its disease. The biological activity of a stressor is therefore an important factor in determining what the subsequent alteration to transcriptomes of gill tissue will be. The fields of immunology and toxicology include (Hansen and Jensen, 2010) many investigations of the transcriptomic alteration of gill tissue in response to insults such as environmental contaminants, infectious disease or physiological stress (Dunier, 1996; Hansen and Jensen, 2010; Maekawa et al., 2019). These publications utilise a number of different techniques for exploration of the altered gene expression of tissues. Progression from the study of individual transcripts through

quantitative polymerase chain reactions (q-PCR) or western blot to high-throughput technologies has allowed researchers to study whole transcriptome responses. This allows the study of which biological pathways are altered in response to stimuli, rather than single genes that may be altered, which most likely provides more biologically meaningful results. As more is discovered about the pathways of response to specific agents of gill pathology, more can be learnt about how best to limit the impact of infectious agents or environmental stressors on gills, either through therapy, legislation, breeding or even genetic modification of aquaculture stocks. Studies have shown that the intensive stocking of aquaculture produced fish (Ellison et al., 2018) as well as farmed fish genetics (Pawluk et al., 2018) and stress of confinement (Uren Webster et al., 2018) all alter the functional transcriptomic response and disease susceptibility of farmed fish.

1.4.2 Dysbiosis

As previously discussed, many infective conditions can impact gill tissue. Negative alteration to the resident microbial community (known as dysbiosis) is also of a concern during gill pathologies. This refers to disadvantageous alterations to adherent microbial populations, where symbionts conferring some advantage to the healthy host are lost and replaced by less useful or even harmful microbes (Carding et al., 2015). Dysbiosis is however hard to characterize, as compositional changes for adaptation are hard to differentiate from dysbiosis and loss of control of the microbiome, at least until a clear disease state becomes apparent. Research into human microbiomes suggests dysbiosis can manifest as a symptom of disease, but can also be associated with the pathology of the disease itself (Menees and Chey, 2018). Distinct microbial signatures as a result of infection are reported and can be helpful in diagnosis and monitoring of disease progression in conditions such as Human Immunodeficiency Virus-1 (HIV) infection, which alters the immune state of its host (Crakes and Jiang, 2019).

Research in terrestrial vertebrates suggests that dysbiosis is often preceded by a stressor that initiates microbial change and results in the loss of important residents or even growth or harmful microbiota (Carding et al., 2015). A complete understanding of what constitutes a ‘healthy’ microbiota is, however, often still unclear. For example, a varied

microbiota is observed between farmed and wild fish, which is attributed to factors such as rearing conditions, diet and genetics (Lowrey et al., 2015; Webster et al., 2018). Identically reared rainbow trout from different genetic stock have been highlighted as having significant variation in microbial communities (Brown et al., 2019), with a demonstrated influence too of stocking density on community composition. Considered alone, apparently healthy individuals within a farmed population are assumed to host a normal and functional microbiome. However, when contrasted to wild counterparts, the artificial rearing environment of farmed fish appears to impair diversity of colonizing organisms (Webster et al., 2018), potentially leading to an adherent community lacking microbial components of importance. The intensive methods of production utilised for farmed fish might then themselves be a risk factor in initiation of dysbiosis in salmonids, and impact susceptibility to disease in these fish. Use of antibiotics is certainly linked with altered microbial community structure, and can allow overgrowth of harmful bacteria through clearance of susceptible commensals (Higuera-Llantén et al., 2018; Pepin et al., 2005). The current thinking in dysbiosis associated variables of salmonids is illustrated in **Figure 1.4**.

'Healthy' microbial communities and alterations to community structure are therefore of interest in understanding the microbiome of salmonids. But what might be considered an 'optimal' microbiome is clearly a complex topic. Determining whether microbial change is for advantageous effect and adaptation (for colonization with microbiota best adapted to an altered ecological niche, for example) or as part of dysbiosis can be problematic to determine. Current research in salmonids has identified several microbes with apparently important symbiotic functions as part of the GIT microbiome, and alterations in the presence or relative abundance of these microbes can be helpful in the appraisal of community change. Bacteria with known pathogenic consequences are also a helpful indicator of negative change, suggesting proliferation of unwanted microbes rather than controlled adaptation. Microbial biomarkers are utilised in research on human microbiomes, to determine stage, prognosis and recovery of various disease states, and might be similarly applied to salmonid health (Carding et al., 2015; Pascal et al., 2017). In this context, the use of pre- and probiotics in fish has

already begun to be explored, and presents an exciting field of research in disease prevention and treatment (Brugman et al., 2018)

Figure 1.4 Factors influencing dysbiosis

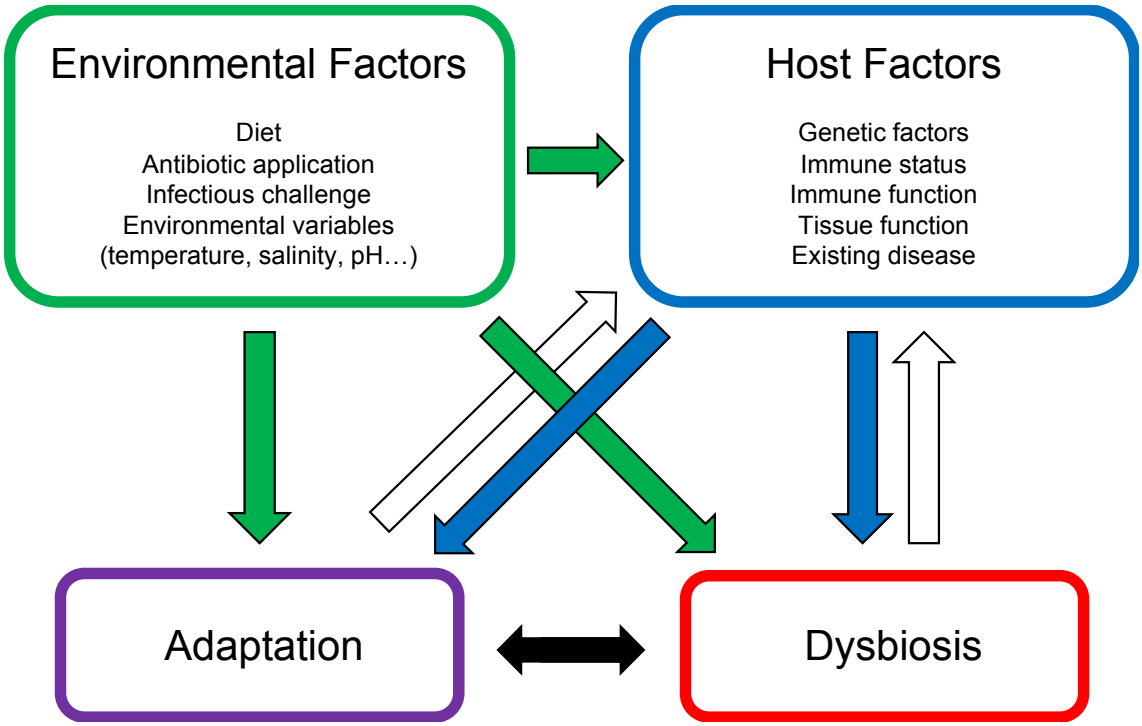


Figure 1.4: *Summarised current research regarding the complex interactions of factors influencing alteration to the microbial communities of salmonids. Adaption and dysbiosis can be problematic to differentiate without a greater understanding of the utility of microbial change.*

1.4.2.1 Investigating microbial communities

Microbial communities and specific taxa can be assessed using a variety of techniques, both sequencing-dependant and sequencing-independent. Culturing represents the original sequencing independent technique for differentiation of bacterial isolates. By growing bacteria on general or specialized media, bacteria within a sample can be cultured and differentiated based on phenotypic characteristics like colony morphology, enzymatic activity or antibiotic resistance (Pazos et al., 1996; Vartoukian et al., 2010;

Zimbro et al., 2009). Unfortunately, culture frequently fails to identify the vast majority of microbial species that can be observed through other methods (Belkin and Colwell, 2005; Joint et al., 2010), and lacks the sensitivity of taxa-assignation possible through sequencing (Cummings et al., 2016; Hiergeist et al., 2015; Mcdonald et al., 2017). Culture of marine bacteria is particularly difficult due to the unknown growth requirements for many taxa. It does however remain an essential step in the description of novel bacterial species (Joint et al., 2010; Schlaberg et al., 2012; Schumann et al., 2009).

Other sequencing-independent methods of microbial characterisation include Ribotyping and DNA-hybridisation. Ribotyping is a technique used historically for differentiating species of bacteria based on fingerprinting of species-specific gel patterns of DNA fragments (Huot and Goldstein, 2008). This technique is used infrequently by modern research, as it assumes unique patterns of DNA fragments within bacterial species and relies upon accurate taxonomic classification of comparison ribotype patterns. DNA-DNA hybridization is a technique requiring comparison of an unknown sample with a known, labelled sample and is still considered the gold stand for identifying bacterial isolates to species level (Busse et al., 2010). DNA mixtures of single strands of known and unknown isolate are hybridized to form hybrid double stranded sequences, with highly similar genomic material binding with higher affinity. This technique is used frequently for determining the taxonomic relationship of samples, with similarity values obtained from multiple DNA-DNA hybridizations used to construct phylogenetic trees (Brenner, 1973; Richter and Rossello, 2009). This technique is though less appropriate for species-level resolution of a truly unknown isolate, as it informs on similarity to a known taxonomic isolate but does not suggest species classification unless the isolate selected for comparison is highly similar. Fluorescence *in situ* Hybridization (FISH) uses oligonucleotide probes that, when applied directly to extracted microbial DNA, bind chromosome material and can be detected by their fluorescence using epifluorescence microscopy. This technique is ideal for isolating specific pathogens in mixed populations, however it differentiates poorly between closely related bacterial isolates, such as *Vibrio* species (Chatterjee et al., 2012).

The majority of modern microbiological research utilises sequencing-dependant methods for exploration of bacterial communities. Taxonomic classification of bacteria can be performed by direct sequencing of PCR products to obtain nucleotide base sequences, either by traditional Sanger or high through put next generation sequencing (NGS) (Klindworth et al., 2013). Large databases of taxonomically classified nucleotide base sequences are available for comparison of unknown isolates, providing a much less subjective method of microbial classification than culture. There exists a great deal of literature regarding bacterial classification using sequencing of the 16S gene of bacterial genetic material; a well-established method of classification (Janda and Abbott, 2007; Weisburg et al., 1991; Woo et al., 2008). This is a highly conserved gene across bacterial taxa, encoding the essential 16S ribosomal subunit. Variation within this gene allows for differentiation of different classifications of bacteria, but the extreme distal portions of the gene remains similar enough to allow universal primer design for PCR amplification (Rheims et al., 1996). Sequencing of the 16S subunit is the basis too of next generation 16S microbiome investigation studies. Next generation studies amplify only a small, variable region of the gene, often the V3/V4 or V1/V2 regions, to obtain a large number of sequence reads. Commonly used Illumina sequencing results in approximately 400bp length amplicons. These sequences can be compared directly against publicly available databases of previously sequenced material for taxonomic classification of mixed polycultures obtained directly from the environment. Next generation sequencing is currently the method of choice in studies investigating diversity of bacterial communities, utilizing relative proportions of different operational taxonomic units to identify differences in alpha and beta diversity in different individuals or environments (Chelius and Triplett, 2001; Cummings et al., 2016; Legrand et al., 2018; Ornelas-García et al., 2018; Reverter et al., 2017). Sanger sequencing remains relevant in a variety specific of applications for microbial investigation, applied for sequencing a variety of bacterial genes in more focused investigations (Beaz-Hidalgo et al., 2015; Hoffmann et al., 2010; Mulet et al., 2009b). This method of sequencing can obtain much larger DNA fragments than next generation sequencing, to obtain the entire 16S gene sequence of approximately 1500bp by a combination of forward and reverse sequencing, or sequence other genes of interest. The drawback of Sanger sequencing is that whilst PCR amplification can be performed

on mixed bacterial genomic material, all amplicons submitted for Sanger sequencing must be of identical base pair sequence to be properly read. This problem can be overcome by sub-cloning the PCR products and sequencing independent amplicons; however this is time consuming and limits conclusions regarding microbial diversity.

Other options for exploration of microbial communities include whole-genome shotgun sequencing, which allows generation of greater length DNA reads, and improved taxonomic resolution over the more often used 16S sequencing approaches (Ranjan et al., 2016), which can suffer from lack of accuracy, especially with short reads (Cooke et al., 2019). This approach is however much more costly, especially when dealing with large sample sizes.

Modern molecular methods are generally favoured for the exploration of diversity of microbes present within a sample, including the commonly used techniques of next generation sequencing of 16S libraries or whole genome shotgun sequencing. Prior to mainstream use of metagenomic techniques, isolation of the entire 16S ribosomal RNA subunit gene through Sanger sequencing was commonly performed for identification of unknown bacteria isolates, and still has utility today. More modern genomic techniques benefit analysis by removing the requirement for isolation and lab cultivation of bacterial isolates. Next-generation sequencing targets shorter, hyper-variable regions of the 16S gene to allow taxonomic classification of entire mixed populations of microbes present within a sample, albeit to a lower taxonomic resolution than can be obtained from Sanger sequencing. Whole genome transcriptome sequencing (RNA-seq) on the other hand allows exploration of the gene expression of complex bacterial communities, providing an expression profile of active bacteria within a sample (Bashiardes et al., 2016). This has the advantage of informing regarding the activity of a bacterial community within a given context. All sequencing-dependant techniques then have utility in exploration of microbial isolates, answering slightly different questions regarding the communities therein.

Depending on the research hypothesis, other approaches can also be taken. Abiotic model species such as zebrafish can provide an excellent avenue of investigation in

understanding the function of microbial isolations *in vivo* (Douglas, 2019; Watts et al., 2012), as well as many other aspects of symbiotic relationships (López Nadal et al., 2020). Fluorescence in-situ hybridisation (FISH) is also a useful tool in many ways. Not only can select microbiota be visualised in-situ as part of descriptive imaging, this technique can be paired with flow cytometry for microbial isolation (Levsky and Singer, 2003; Liu et al., 2011).

1.4.3 Gross and histological assessment

Alterations in response to gill tissue damage and stress can occur as part of systemic physiological change, such as increasing the extent and rate of lamellar perfusion through increased myocardial effort during hypoxia (Farrell et al., 1980). There will also be cell and molecular-level changes, impacting tissue structure and morphology. Changes to the ion content of the external environment, such as occurs during transition from fresh to salt water, can elicit changes to the cellular composition of the chloride cells in gills, with cell migration along lamellar surfaces (Perry, 1998; Sardet et al., 1979). Altered cell numbers can be an indicator of gill adaptation to external stressors, as can infiltration of inflammatory cells as part of the immune response. More extreme alterations can also be observed. Commonly reported structural changes in response to stress include lamellar fusion, hyperplasia and loss of the normal surface structure. Cell necrosis, epithelial lifting and hypertrophy are also commonly observed in gills that are considered to have compromised functionality (Wolf et al., 2015). Unfortunately, the majority of these alterations are non-specific changes that occur in gill tissue in response to a multitude of causes (Flores-Lopes and Thomaz, 2011). Infectious organisms can occasionally be visualized in tissue sections, or characteristic changes as part of their pathology observed, such as pseudocyst formation in gill tissue with AGD. These observations assist in presumptive diagnoses. The majority of histologically observable alterations are non-specific though, occurring to varying degrees and in either localized or extensive fashion.

Many of these structural alterations that commonly occur in gills with tissue insult also have apparent negative consequences for fish (Harper and Wolf, 2009). Alterations to

the gill architecture like fusion of lamellae along a filament surface are a common finding in gill disease. Fusion of entire filaments can even occasionally occur in more extreme examples of gill disease (Mitchell et al., 2012). This results in a reduced overall surface area of the gill tissue. Research suggests, when observed in conjunction with infective disease, this response may be as part of an adaptation for exclusion of microorganisms. It does, however, have the negative consequence of reduced area for oxygen exchange. Increased mucus production has also been documented in gills experiencing traumatic insult, presumably to provide increased surface protection for the tissue. Mucus contains immune cells and antimicrobial proteins, to prevent pathogen entry (Lumsden et al., 1994). Unfortunately, a preponderance of mucus negatively impacts the ability of gills to perform respiration efficiently (Mitchell et al., 2011b).

Gill tissue is much more complex than can be observed with the naked eye through gross assessment, although visual appraisal is useful in the broad detection of abnormalities. Gill surface topography can be assessed in detail using scanning electron microscopy, however, techniques such as histopathology are required to visualize sub-surface anatomy and changes such as alterations to cell types within gill tissue. Histological preparation of gill tissue, through sectioning, staining and fixation allows microscopic structures to be observed. Various stains can be utilized, as well as assessment of immunohistochemistry or *in situ* hybridisation for specific pathogenic agents. Although histopathology of gills is not as reliable in specific disease diagnosis as the available molecular techniques, it does provide excellent insight into tissue integrity, and the outcome of disease processes. General assessment of gill changes and functional impairment can be obtained through simple haematoxylin and eosin (h&e) staining of sections, but proper appraisal of these sections is very time-consuming and can be subjective. Scoring systems for semi-quantitative categorization of histology simplify the appraisal of gill tissue, providing a framework for comparison across individuals, although they are not without drawbacks. Adherence to a set of parameters for classifying gill pathology potentially excludes meaningful change from inclusion in final results. They do however allow a more rapid and consistent method of classifying pathology within a dataset.

1.5 Aims of this thesis regarding the onset and pathogenesis of gill disease

There remain many unanswered questions regarding the causes of disease in the marine stage of aquaculture production of salmonids. Although extensive research exists on the topic, much is still to be learnt about the complex and apparently often mixed pathologies of gill disease. When observed in farmed fish, gill pathologies are often described as syndromes rather than being due to a causative agent. Microbes considered causative agents of diseases in some individuals can be isolated from other entirely healthy appearing fish, even as apparent key components of the healthy microbiome in the case of epitheliocystis-associated *Candidatus Branchiomonas* in rainbow trout (Brown et al., 2019). Even the readily diagnosable infection AGD has been suggested to occur due to a confluence of other factors, such as altered microbial communities of the gill tissue (Bowman et al., 2004). Clearly much is still to be learning regarding the predisposing and concomitant factors influencing gill disease onset and progression. Farmed fish appear particularly susceptible, likely due to the combined influence of genetic and environmental factors, and so are of particular interest in this field of study, never mind their major economic importance.

Non-infectious water-borne irritants or environmental biological events associated with gill disease, such as algal or jellyfish blooms, are not species-specific, and pose a threat to all finfish production. It is well understood that these harmful environmental organisms can illicit damage to gill tissue, however, little is known about their association with complex gill pathologies. Clearly high concentrations of these organisms cause tissue damage and even mass mortalities in farmed fish (Bosch-Belmar et al., 2016a; The Scottish Government, 2006), but little information is available regarding their subclinical impact, and association with other pathologies. It therefore seems relevant that the impact of these organisms on gill tissue should be explored, to determine their role (if any) in complex gill pathologies. Populations can fluctuate massively, with potentially significant but as yet undetected impact on farmed fish. This thesis sought to address the areas of research highlighted above, identified as currently lacking in their ability to inform the production of salmonids. The aim was for exploration of the impact of harmful environmental organisms on gill health, their

potential association with subsequent gill disease and alterations to microbial communities. The potential for vector transmission of infections bacteria by cnidarian jellyfish species was explored through identification of any harmful microorganisms within the cnidarian tissue microbiome to understand the role these organisms might play in initiation of gill disease. The transcriptomic response of rainbow trout gill tissue to harmful algal organism *Prymesium parvum* was also investigated. These toxic algae have been suggested as directly traumatising gill tissue however the mechanism of damage and tissue response of fish was previously unexplored. This research therefore represents the first study of alteration in genetic expression of fish gill tissue to toxin production phytoplankton, and sought to provide useful insight into the mechanisms of gill response. Research sought to address the important question of the impact of phytoplankton on fish immunity, and the potential for these organisms to impair or otherwise alter the transcriptome response in complex gill disease. It was decided also to study the microbial community structure of farmed Atlantic salmon gills and attempt to understand the factors that influence the alteration and potential dysbiosis of these populations. The aim was to understand the impact of several variables on microbial community composition during a production cycle, including cnidarian and phytoplankton population exposure. Much of the existing research into microbial communities explores dysbiosis, but few focus on distinguishing advantageous adaptation and the impact of 'routine' on-farm treatments.

Chapter 2

2. Methods

This chapter describes the main methodologies including experimental and initial data process methods of this thesis, as well as design and implementation of the collection of samples for chapters 5 and 6. It is intended as a reference, with referral made to relevant sections in subsequent results chapters.

2.1 Basic laboratory techniques and working conditions

Surfaces and pipettes were cleaned with 70 % (v/v) ethanol (Sigma-Aldrich) for all experimental work to reduce contamination with environmental bacteria. Work related to chapters 5 and 6 for investigation of the microbiome was conducted where possible in the laminar flow hood, and next to a Bunsen when performed bench-side. Standard sterile technique (Cappuccino and Sherman, 2014) was used for all tissue handling within the laboratory. Tools and surfaces for use in handling tissue for chapter 4, transcriptome analysis, were additionally cleaned using RNase AWAY surface decontaminant solution (ThermoFisher Scientific) to prevent contamination or degradation of samples. Autoclaved milli-Q water (Millipore) was used in all molecular biology procedures unless otherwise stated. Finally, all plasticware was certified sterile on purchase, with use of filtered pipette tips (Eppendorf) for the majority of methods.

2.1.1 Gel Electrophoresis

Agarose gels were made by dissolving agarose powder (Bioline) in 1x Tris-Acetate-EDTA buffer (TAE) (ThermoFisher Scientific). Gel mixtures were melted by microwave (Daewoo) and ethidium bromide added once the mixture was cooled for a final concentration of 0.5 µg/ml. Gels were poured in electrophoretic apparatus with an 8-piece comb and allowed to solidify for minimum 20 minutes before being submersed in TAE for running. Loading dye and Generuler DNA ladder (ThermoFisher Scientific) were used when loading gels. Gels were run at varying voltage depending on size of

expected product for separation of nucleic acids. Ethidium Bromide-bound bands were visualised under ultraviolet light using Benchtop 2UV transilluminator (BioDoc).

If required, bands of the appropriate size were cut from the gel using a sterile blade and purified using Isolate II PCR + Gel kits (Bioline) following the manufacturer's instructions.

2.1.2 DNA sub-cloning

Sub-cloning was performed using the pGEM-T Easy vector (Promega labs) using the manufacturers recommended protocol modified to utilise half the amount of vector and with the insert quantity adjusted accordingly. Plasmids were transformed into the XL1-blue or XL10 blue strains of *Escherichia coli* (Agilent Technologies) via standard heat shock procedures, and grown at 37 °C overnight on LB agar plates containing 50µg/ml of Ampicillin (Fisher Bioreagents, ThermoFisher Scientific) with each plate spread with 40 µl of 20 mg/ml X-gal (Sigma) and 4 µl of 200mg/ml Isopropyl ß-D-1 thiogalactopyranoside (IPTG) (Sigma) to enable blue-white selection of insert-containing plasmids. Independent bacterial colonies were removed from each plate and grown in LB broth culture with 50 µg/ml Ampicillin (ThermoFisher Scientific) at 37 °C overnight with shaking. Plasmid DNA was isolated using the DNA miniprep kit (Qiagen) following the manufacturer's instructions.

2.1.3 Quantification and quality control

2.1.3.1 Nanodrop

DNA concentration and 260/280 and 260/230 ratios were measured using Nanodrop 1000 spectrophotometer (ThermoFisher Scientific) for DNA and RNA.

2.1.3.2 Qubit

Qubit dsDNA BR and Qubit dsDNA HS Assay Kits (ThermoFisher Scientific) were used according to manufacturer's instructions with Qubit 4.0 Fluorometer (Invitrogen, ThermoFisher Scientific) for quantification of DNA extraction concentrations at various stages of next generation sequencing (Section 2.6). Broad Range (BR) kits were utilized to assess the concentration of initial DNA extractions and final index PCR reactions (Sections 2.6.1 and 2.6.4). High Sensitivity (HS) kits were used to assess the concentration of genomic material in initial amplicon reactions (Section 2.6.2). Reagent buffer mixtures and sample reactions were prepared for both according to manufacturer's recommendations.

2.1.3.3 Bioanalyser

RNA solution concentrations were assessed using Bioanalyser RNA 6000 Nano Kits (Aligent) with 2100 Bioanalyser (Aligent) according to manufacturer's instructions following RNA extraction and pooling. This analysis informed regarding the purity & integrity of obtained RNA prior to use in microarray.

2.1.3.4 Pilot studies

Several pilot experiments were employed to ensure the efficiency of molecular techniques in obtaining results prior to use of all samples in the relevant study. Generated results were used to inform decision making in protocol adaptation to optimise research results. Details of specific pilot extractions, sequencing and analysis can be found in the relevant results chapter method sections.

2.2 Sample collection

2.2.1 Collection of material for exploration of the microbiome of gill tissue and associated pathology

Samples were obtained from Atlantic salmon in the marine stage of aquaculture production on the West Coast of Scotland from a single Scottish Sea Farms (SSF) facility (Loch Spelve, 56.374760, -5.768232). Fish were of variable age during the study, starting in May 2017 with approximately 1-year old smolts (average weight 84.8 g) through to pre-harvest fish (average weight 3463 g) in June 2018. The sampling regime involved removal of 12 fish at each visit. Bimonthly sampling visits for collection of fish were supplemented by additional visits for additional sampling following on-farm events considered to be of interest with regards an impact on bacterial populations. Scheduled sampling occurred at approximately 60-day intervals, subject to weather and the schedule of SSF staff. Sampling was conducted on dates detailed in **Table 2.1**. Samples were obtained exclusively from a single SSF location and single sea cage (cage 1). Cage 1 was selected as this was the population of fish feeding most enthusiastically during the first sampling visit, facilitating the easiest capture. Fish were obtained using food as an incentive, to encourage fish to the water surface, and then using a crowing net to isolate a group of fish. A small hand net was then utilized to capture and removed individual fish until the desired total of 12 was obtained.

A total of 132 Atlantic salmon were sampled over the entire fieldwork period. True randomization of fish selection cannot be confirmed due to the method of capture, as fish of impaired health status, such as those suffering from extreme gill disease, are known to be less likely to feed, spending the majority of their time in the lower water column (Brown et al., 2011). It was, however, not possible to collect the fish from the lower levels of the marine cage without significant cost and disruption to the entire population. The sampling regime was consistently maintained throughout the fieldwork period, ensuring consistency if not true randomization of stock selection. Captured fish were removed from the sea cage and placed in a water bath of approximately 500 mg/L

3-aminobenzoic acidethyl ester methanesulfonate (MS-222) for euthanasia by SSF staff (Readman et al., 2017; Topic Popovic et al., 2012). The capture and euthanasia of fish was performed by SSF employees under the auspices of their ethics and licensing. This methodology was approved by the Animal Welfare and Ethics Committee, University of St Andrews.

Table 2.1 Visit number and date

Sample Visit	Date
1	17.05.17
2	26.06.17
3	26.07.18
4	07.09.17
5	24.09.17
6	24.11.17
7	30.11.17
8	31.01.18
9	8.03.18
10	23.03.18

Table 2.1*: Visit numbers and dates for collection of gill tissue from SSF sampling site. Visits 1, 3, 5, 6, 8 and 10 were conducted as part of the sampling regime, with additional sampling performed to coincide with on-farm management interventions or events.*

2.2.1.1 Sample fixation

Once fish were determined to be dead (with confirmed cessation of movement and reaction to stimuli, respiratory arrest and loss of the vestibulo-ocular reflex) (Leary et al., 2013), a brief external post-mortem assessment was performed, with focus on gross gill pathology. Sampling was then performed immediately to prevent autolysis of tissue samples (George et al., 2016). Further post-mortem assessments were later made of individual fish. Details of the weight, appearance and any notable pathology were recorded however have not been included pending approval from the producer. Fish were individually identified using a numbering system. For example, fish 3F6 was obtained on visit 3, and was the sixth individual sampled.

Left side first gills were fixed for analysis of the microbiome. Half of material removed was fixed in 25ml absolute ethanol (ThermoFisher Scientific), and half was fixed in 25ml RNAlater solution (ThermoFisher Scientific). Left side second gill arches were fixed in 40ml neutral-buffered formalin (Cellstor, CellPath) for histological analysis. Tissue samples obtained are summarized in **Figure 2.1** and **Table 2.2.** Tissue sections were approximately 1 cm x 1 cm x 1 cm in size and included both cartilage and lamellar gill tissue (**Figure 2.2**). No effort was made to wash or dry tissue prior to placing in fixative in order to avoid disruption of the mucus layer and its associated microbiome. Ten each of ethanol and RNAlater fixed swabs were obtained from the right side first gill, across area highlighted in **Figure 2.2**. Fixed material was maintained at ambient temperature for approximately 24 hours before cold storage on return to laboratory facilities. Swabs were collected by use of a sterile cotton swab applied to an equivalent area of gill surface excised for biopsy fixation. Instead of excision of tissue on right side however, a swab was passed progressively over the entire identified region of tissue and placed in appropriate fixative.

Table 2.2 Tissue source and fixative

Sample	Size	Site
Ethanol fixed tissue	$1cm^3$	G1, Left side
RNAlater fixed tissue	$1cm^3$	G1, Left side
Formalin fixed tissue	$1cm^3$	G2, Left side

Table 2.2: *Tissue origin, sample size and fixation media for gills sampled from every fish. Several fish were additionally sampled on the right side, first arch by swabbing.*

Figure 2.1 Gills utilised in sample collection

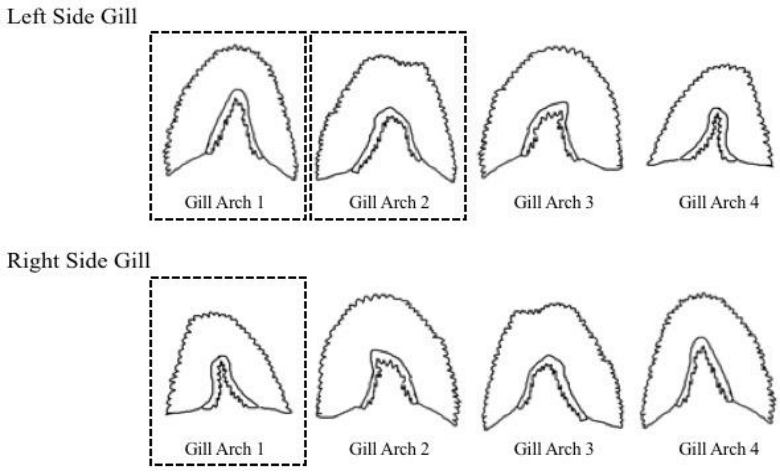


Figure 2.1*: Gills removed from euthanised fish for biopsy sample collection. First and second gills were utilised from the left side of the fish, facing forward, and the first gill on the right was also sampled from in a number of individuals.*

Figure 2.2 Area of excision for tissue fixation

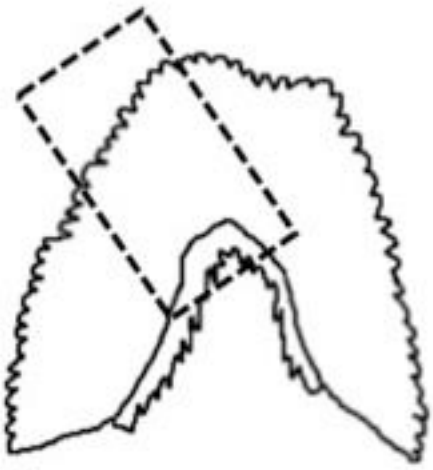


Figure 2.2: *Area of tissue utilised for biopsy excision from RNAlater fixed gills and subsequent DNA extraction. Tissue from the opposing side of the arch was removed for ethanol fixation.*

2.2.1.2 Histology

Formalin fixed tissue was embedded, sectioned and stained by the external contractor The Fish Vet Group (Benchmark Animal Health). Simple haematoxylin and eosin (H&E) stain was used to facilitate assessment of microscopic pathology within gill tissue. Histological assessment was performed using a light microscope at various magnification levels to determine the details and extent of any abnormal tissue presentation. Each sample (132 total) was initially analysed at x4 and x10 magnification to provide general impressions of overall quality of the section. Appraisal of tissue structure and presence of any indicators of pathology or disease was then performed at x20 and x40 magnification, with brief notes taken of the features of pathology or unusual findings. An existing scoring system for semi-quantitative classification of samples was applied to H&E stained histological sections to obtain a numerical score regarding degree of gill disease (Mitchell et al., 2012) (**Table 2.3**) . Scoring system assigns a score of 0-3 for key pathological findings depending on their overall presence and severity within gill tissue, and additional score of 0-1 depending on absence/presence of other listed parameters. Gills then achieved a numerical score based on the total obtained observable histological change. Scoring was performed blind to prevent group bias, and only by the author to ensure multiple observer bias did not impact results. Repeat analysis of a subset of samples will be performed for eventual publication of results, however only analysis performed by the author is included in this thesis.

Table 2.3 Histological scoring criteria

Pathology	Type of indices	Score	Levels	Description
Lamellar hyperplasia	Index	0 - 3	None (0)	No hyperplastic change
			Mild (1)	Low level of focal hyperplasia (<10% gill)
			Moderate (2)	Widespread or multifocal (10 – 50%)
			Severe (3)	Extensive hyperplastic change (>50% gill)
Lamellar fusion	Index	0 - 3	None (0)	None or never minor lamellar fusion
			Mild (1)	Occasional fusion of filaments (<10%)
			Moderate (2)	Multifocal areas of fusion (10 – 50%)
			Severe (3)	Extensive (>50%) with loss of normal architecture
Cellular anomalies	Index	0 - 3	None (0)	None
			Mild (1)	Scattered apoptotic or necrotic cells with/without focal sloughing
			Moderate (2)	Multifocal areas of apoptotic or necrotic cells with/without multifocal sloughing
			Severe (3)	Widespread necrosis with/without sloughing, visible throughout section
Lamellar oedema	Index	0 -3	None (0)	None
			Mild (1)	Epithelio-capillary separation with proteinaceous fluid in the space (<10%)
			Moderate (2)	Multifocal oedema (10 – 50%)
			Severe (3)	Widespread oedema (>50%)

Pathology	Type of indices	Score	Description
Inflammation	Ancillary	0/1	Presence of inflammatory cells outside the blood vessels
Eosinophilic granular cells	Ancillary	0/1	Higher than normal eosinophilic granular cells outside the blood vessels
Circulatory disturbance	Ancillary	0/1	Thrombi, telangectasis, stasis (>10% of tissue effected, not associated with artefact)
Cellular hypertrophy	Ancillary	0/1	Hypertrophic change to epithelial or other gill cell types
Bacteria (Tenacibaculum)	Ancillary	0/1	Matts of filamentous bacteria on lamellar surfaces
Bacteria (Epitheliocystis)	Ancillary	0/1	Intracellular infection
Bacteria (other)	Ancillary	0/1	Unidentified bacterial plaques
Neoparamoeba	Ancillary	0/1	Presence of amoeba on gill tissue
Costia	Ancillary	0/1	Presence of Costia protist on gill tissue
Trichodina	Ancillary	0/1	Presence of Trichodina on gill tissue
Other parasites	Ancillary	0/1	Presence of other parasites on gills

***Table 2.3**: Histological scoring criteria from previous gill pathology publication (Mitchell et al., 2012). Index criteria are scored from none to severe (0 – 3), and ancillary criteria are considered as adjunct indicators of gill disease, scored on presence/absence (0/1).*

2.3 DNA and RNA extractions

2.3.1 Microbial DNA extraction from cnidarian tissue

Extraction of microbial genomic material from Cnidarian tissue samples was performed by previous PhD student, Anna Kintner (AK). Samples for this thesis were obtained in the form of frozen pellets of microbial DNA, extracted from bacterial colonies by crude boiling approach. Specifics of sample collection and treatment by AK can be found described in the relevant results chapter (Chapter 3, Section 3.3.1).

2.3.2 Microbial DNA extraction from salmonid gills

DNA was extracted from 8-15 mg of RNAlater fixed gill tissue sections from each sampled fish using a modified protocol for DNeasy Blood and Tissue extraction kit (Qiagen). Tissue sections physically disrupted to facilitate digestion prior to addition of 180 µl of ATL buffer (DNeasy Blood + Tissue kit, Qiagen) and 20 µl Proteinase K

(Qiagen). This was performed in duplicate for each fish sample. Swabs were air dried in a laminar flow hood before use. A sterile scalpel blade was used to scrape swab surfaces to remove adherent material before duplicate extractions in 180 µl of ATL buffer (DNeasy Blood + Tissue kit, Qiagen) and 20 µl Proteinase K (Qiagen) were performed for each swab. Entire environmental samples were centrifuged at 4200 rpm for 10 minutes and the majority of supernatant removed before being vortexed and transferred to two microcentrifuge tubes. These were centrifuged at 13,000 rpm for ten minutes. Supernatant was removed and discarded, with the remaining liquid evaporated from the sample by air drying within a laminar flow hood. Separate samples were then treated with addition of 180 µl of ATL buffer (DNeasy Blood + Tissue kit, Qiagen) and 20 µl Proteinase K (Qiagen).

Samples were incubated at 56 °C with agitation by vortexing at 15 minute intervals for the first hour, followed by an overnight (12 hours) incubation at 56 °C without agitation. Following digestion, samples were briefly vortexed to ensure complete digestion and mixing of sample, before addition of 2 µl RNAase enzyme (Ambion) and gently mixed by inverting. 250ul of phenol and chloroform isoamyl alcohol mixture in 25:24:1 ratio, pH 7 (ThermoFisher Scientific) was applied to samples and allowed to sit for 60 seconds. Samples were then gently mixed by inverting before centrifugation was performed at 13000 rpm for 15minutes. The aqueous phase containing genomic material was then removed and placed in a fresh sterile microcentrifuge tube. 250 µl of chloroform was then applied to the aqueous phase, gently mixed by inverting, and centrifuged for 15 minutes at 13000 rpm. The aqueous phase containing DNA was collected and placed in a fresh microcentrifuge tube. DNA extraction was then completed using the DNeasy Blood & Tissue kit (Qiagen) according to manufacturer's guidelines. Samples were eluted in 200 µl of buffer AE (Tris) as recommended following an incubation of the column membrane for minimum 120 seconds at room temperature. A spin column control was obtained by use of the DNeasy kit without tissue inclusion for each round of DNA extractions.

Initial extractions from RNAlater fixed tissue contained high levels of protein contamination, with poor performance in pilot study polymerase chain reactions (PCRs), prompting the inclusion of the phenol/chloroform step. Samples fixed in absolute ethanol (Sigma-Aldrich) were not utilised due to common failure of DNA extractions.

DNA concentration and 260/280 and 260/230 ratios were measured using a Nanodrop 1000 spectrophotometer (ThermoFisher Scientific). Extractions with a DNA concentration < 20 ng/µl were repeated. Extracted samples were also run on a 1 % agarose gels with ethidium bromide to observe the integrity of extracted DNA. Fragmentation of DNA was observed within initial sample gels, prompting the removal of vortexing from the phenol/chloroform extraction protocol, and replacement instead with gentle mixing. Observed fragmentation was greatly reduced by this substitution.

2.3.3 RNA extraction from salmonid gills

Gill tissue was obtained from juvenile rainbow trout experimentally exposed to toxin producing phytoplankton in a challenge trial conducted at the Aarhus University, Denmark. The design and implementation of this challenge trial was not conducted as part of this PhD, and so available details are provided briefly in the relevant results chapter (Chapter 4, Section 4.3.1).

Individual gills of approximately 100mg were removed from RNAlater and gentle blotted to remove excess liquid. Tissue was then lysed and homogenized in 1 ml chilled TRIzol reagent (ThermoFisher Scientific) and 3 mm tungsten carbide beads with the Tissue Lyser II Disruption System (Qiagen) according to the TRIzol protocol for RNA extraction (Invitrogen, ThermoFisher Scientific). Lysates were incubated for 5 minutes at room temperature to allow the melting of nucleic acid-protein complexes before addition of 200 µl of chloroform and incubation for a further 5 minutes. Samples were then centrifugation for 15 minutes. The aqueous phase was then removed from samples and transferred to fresh microcentrifuge tubes. 500 µl Isopropanol (Sigma) was added to the aqueous phase and allowed to precipitate for 10 minutes before being centrifuged

for 10 minutes. Supernatant was then discarded, taking care to remove no to disturb the RNA pellet. All centrifugation steps were performed at 12,000 and 4 °C (Eppendorf Centrifuge 5424) unless otherwise stated. All incubation steps were performed at room temperature. RNA pellets were washed twice using 75 % ethanol (Sigma-Aldrich). This involved application of 1 ml ethanol, brief vortexing and centrifugation for 5 minutes at 7500 × g in the chilled centrifuge before removing ethanol by micropipette. Pellet was then allowed to air dry for approximately 5 – 7 minutes before resuspension in 30 µl Ultrapure RNase-free water (Invitrogen) within a 55 °C heat bath for 10 minutes.

2.4 Polymerase Chain Reactions for Sanger sequencing

Polymerase Chain Reaction (PCR) was performed using archived genomic material from AK to assign taxonomic classification to cnidarian-derived samples. Primers were ordered and obtained from (ThermoFisher custom, ThermoFisher Scientific) and suspended in Autoclaved milli-Q water (Millipore) at a concentration of 100 µg/ml. Unless otherwise stated working stock concentration of primers were 25 µg/ml.

2.4.1 16S PCR

PCR amplification of the 16S genomic region of monoculture-derived DNA extracts were performed using universal primers 20F (5' -AGAGTTTGATCATGGCTCAG-3') and 1500R (5' -GGTTAC-CTTCTTACGACTT-3') (Weisburg et al., 1991) in 25 µl reactions. Reactions contained 2ul each of 20ul/ml (1pmol) forward and reverse primers, 1µl of approximately 100 ng/µl concentration genomic material, 10 µl Autoclaved milli-Q water (Millipore), 0.5 µl MyTaq DNA polymerase (Bioline) and 14.5 µl of nucleotide containing MyTaq Reaction buffer (Bioline) as a modified protocol (Cepeda et al., 2003). Cycling conditions were an initial preheating at 95 °C for 5 minutes, followed by 28 cycles of denaturation (95 °C for 30 seconds), annealing (57 °C for 30 seconds) and extension (72 °C for 60 seconds) and a final extension step consisting of 5 minutes at 72 °C.

2.4.2 *GyrB* PCR

For samples of genus *Aeromonas*, extracted DNA was subjected to PCR using primers GyrB3F (5'-TCCGGCGGTCTGCACGGCGT-3') and GyrB14R (5'-TTGTCCGGGTTGTACTCGTC-3'), amplifying an approximately 1100 bp fragment of the *GyrB* gene (Yáñez et al., 2003). Reactions were performed in accordance with the previously described protocol by Yáñez et al, only with modified volumes for a final reaction volume of 25 µl. The reaction mixture was subjected to 35 cycles of recommend thermocycler conditions (Yáñez et al., 2003).

2.4.3 *rpoD* PCR

Samples identified as being from the genus *Pseudomonas* were subjected to PCR using primers 70F (5'-ACGACTGACCCGGTACGCATGTAYATGMGNGARATGGGNACNGT-3') and 70R (5'-ATAGAAATAACCAGACGTAAGTTNGCYTCNACCATYTCYTTYTT-3') (Yamamoto et al., 2000). Use of these primers for amplification of nucleic acid sequence from *Pseudomonas* was performed as previously described (Mulet et al., 2009a), modified for a final reaction volume of 25 µl. Thermocycling conditions were an initial denaturation period at 94 °C for 5 minutes followed by 32 cycles of amplification (denaturation was performed at 94 °C for 1 minutes, primer annealing was performed at 55 °C for 1 minutes, and primer extension was performed at 72 °C for 1 minutes), as a slightly modified protocol. A final elongation step was carried out at 72 °C for 10 minutes.

2.4.4 Intergenic-Spacer Region (IGS) PCR

Samples from the genus *Vibrio* were subjected to PCR using primers G1F (5'-GAAGTCGTAACAAGG-3') and L1R (5'-CAA GGCATCCACCGT-3') initially described in 1993 (Jensen et al., 1993), targeted to the 16S-23S Intergenic-Spacer (IGS) region. A 25 µl PCR reaction was conducted according to previously published methodologies (Pizarro et al., 1996; Wong and Lin, 2001) but with modified

thermocycling conditions of 5 minutes 95 °C, and 36 cycles of 30 seconds 95° C, 30 seconds 56 °C and 30 seconds 72 °C, with a final extension of 5 minutes 72 °C.

2.5 Sanger sequencing

PCR clean-up was performed prior to sequencing using the BigDye Terminator v3.0 Reaction Kit (ThermoFisher Scientific) according to manufacturer's instructions. Samples were then delivered to either the Department of Zoology sequencing service, University of Oxford, or the Eurofins sequencing service for sanger sequencing reactions. Products of 16S and *GyrB* PCR reactions were sequenced using the original PCR primers 1500R and GyrB3F respectively. Products of *rpoD* amplification were sequenced with specific sequencing primer 70Fs (5'-ACGACTGACCCGGTACGCATGTA-3') (Yamamoto et al., 2000). For inserts from within plasmids obtained through sub-cloning (Section 2.1.3), sequencing was performed with the primer T7 (ThermoFisher Scientific).

Sequencing results retrieved from external providers as sequence files were processed using the program Geneious 9.0.5 (https://www.geneious.com) for removal of primer binding sites and plasmid vector sequence as well as assessment of electropherograms.

2.6 Next generation sequencing

Next generation amplicon generation and library preparation was performed largely in accordance with the Illumina Metagenomic sequencing library preparation protocol (Illumina, 2013). Brief details of the steps and any modifications are provided below.

2.6.1 Amplicon generation

Accurate quantification of DNA concentration of both extractions from every sample were obtained using Qubit dsDNA BR Assay kit (ThermoFisher Scientific) according to manufacturer's instructions with Qubit 4.0 Fluorometer (Invitrogen, ThermoFisher Scientific). A stock 45 ng/ ul stock solution of genomic material from each sample was

then generated by normalisation and pooling of duplicate extractions. Primers 341f (5'-**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**CCTACGGGNGGCWGCAG) and 805r (5'-**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**GACTACHVGGGTATCTAATCC) (Klindworth et al., 2013; Muyzer et al., 1993) (TruSeq) were then utilised for amplification of the V3-V4 region of microbial genomic material and attachment of overhang adaptors (overhang adaptor sequence in bold). Amplicon PCR's were performed in triplicate, using 25 ng of pooled template DNA, 5 pmol of each primer, and 0.5 units of KAPA HiFi HotStart ReadyMix (Roche) in a 25 µl reaction. Included were input material-free control PCR reactions and negative spin columns controls from the relevant batches. Thermocycler conditions were as follows: Initial denaturation at 95 °C for 3 minutes, followed by 27 cycles of 95 °C for 30 seconds, 55 °C for 30 seconds, 72 °C for 30 seconds, and a final extension at 72 °C for 5 minutes.

2.6.2 Cleaning

PCR products from amplicon generation were cleaned using AMPure beads according to the protocol, utilising 20 µl of AMPure XP beads per sample (Agencourt, Beckmann Coulter), and duplicate 200 µl washes using 80 % ethanol (Sigma-Aldrich). Samples were resuspended in 25 µl of 10 mM Tris pH 8.5 buffer. Resuspension was performed in a lower than recommended volume to maximize genomic DNA yield and concentration. An additional qubit assessment was then performed using Qubit dsDNA HS Assay Kit to quantify amplified genomic material prior to normalisation and pooling of triplicate reactions for each sample to a DNA concentration of 1 ng/µl.

2.6.3 Index PCR

Index PCR reactions were performed then for attachment of Illumina sequencing adapters and dual indices (Nextera XT Index Kit, Illumina). The index PCR was performed in the following volumes: 5 µl of primer each from the Nextera XT kit's A and D in a unique combination for each sample, 25 µl of 2x KAPA HiFi HotStart

ReadyMix (Roche) and 15 ng total of pooled template DNA. This protocol was modified from the Illumina recommended methods by removal of 10 µl of PCR grade water from the reaction and inclusion instead of 15 µl of sample. Final 45 µl reactions were treated to the following conditions: Initial denaturation at 95 °C for 3 minutes, followed by 8 cycles of 95 °C for 30 seconds, 55 °C for 30 seconds, 72 °C for 30 seconds and a final extension at 72 °C for 5 minutes.

PCR products were again cleaned using AMPure XP beads (Agencourt, Beckmann Coulter) similar to Section 2.6.2 but this time using 56 µl of AMPure XP beads (Agencourt, Beckmann Coulter) per sample. Samples were eluted in 25 µl of 10 mM Tris pH 8.5 buffer per manufacturers direction to obtain amplicons of approximately 630 bp in an average final concentration of 50 ng/µl. Concentration of DNA was assessed using Qubit dsDNA BR kit (ThermoFisher Scientific) according to manufacturer's instructions with Qubit 4.0 Fluorometer (Invitrogen, ThermoFisher Scientific) (Section 2.1.3.2).

2.6.4 Library preparation

Index PCR products were normalised and pooled to a final library concentration of 4nM. 5 µl of pooled library was then denatured using 0.2N 5 µl freshly prepared NaOH before being briefly vortexed and centrifuged the sample solution at 2000 g at room temperature for 1 minute. Following five minutes of room temperature incubation, HT1 hybridization buffer (Illumina) was applied as per manufacturer's recommendations. Denatured library was diluted to a final concentration of 3pM. PhiX control (Illumina) was similarly prepared.

Prepared library and PhiX were combined to achieve an overall 20% PhiX spike-in. Mixture was denatured 96 °C for 120 seconds, followed by 6 minutes in an ice bath immediately prior to loading the already defrosted MiSeq v3 reagent cartridge (Illumina). The spiked library was then sequenced on a MiSeq (Illumina) using the 600-cycle MiSeq reagent kit v3 (Illumina) according to the manufacturers protocol (300 bp paired-end reads).

2.6.5 Metagenomics workflow

Demultiplexed next generation data from the sequencing of prepared libraries was processed with the assistance of Adam Wyness (AW). The open source DADA2 (Callahan et al., 2016) pipeline was utilised within Qiime2 v2019.2 (Bolyen et al., 2019; Caporaso et al., 2012) for filtering poor quality results and trimming forward and reverse reads based on Phred scores. Dereplication of sequences was also performed. The DADA2 algorithm for modelling error rate was used at a max error rate cut off of 2. Paired end reads were then merged, and chimeras removed through DADA2 for a final table of ribosomal exact sequence variants with total counts.
Amplicon Sequence Variants (ASVs) were obtained in place of OTUs in this workflow. Taxonomy was assigned to results using the SILVA 128 reference database (13.8 version) (Quast et al., 2013). Sequences assigned to chloroplasts, archaea, mitochondria and reads unassigned below kingdom level were then removed for generation of the final dataset for Beta-diversity analysis. Alpha-diversity metrics were calculated for treatment medians of a rarefied dataset (1200) where only treatment groups that reached the rarefaction curve plateau were included.

2.7 Microarray experiments

Following RNA extraction (Section 2.3.3) and determination of sample concentration (Section 2.1.3.1), RNA samples were pooled to achieve four biological replicates for each clinical treatment group (with 16 fish represented in total for each treatment). Each pool containing 10 µg from four fish (40 µg total). Each pool was then subsampled to create a common control with equimolar contribution from all 64 fish. Purity & integrity of RNA solutions were determined using Bioanalyser RNA 6000 Nano Kits (Aligent) with 2100 Bioanalyser (Aligent) as detailed above (Section 2.1.3.3)

2.7.1 Amplification and labelling for two-colour experiment

Microarray preparation was performed as described previously (Castro et al., 2015). Briefly, antisense amplified RNA (aRNA) was generated from RNA pools (16 in total)

using the MessageAmp II aRNA Amplification Kit (Ambion) following manufacturer's instructions (Ambion, 2011). This kit is used to first synthesise cDNA by reverse transcription of RNA, before a second cDNA strand is synthesised. The purified cDNA product of this is then used to generate multiple copies of aRNA with incorporated amino allyl UTP, to facilitate fluorescent labelling. Following purification of aRNA and quality check (Section 2.1.3.1), aRNA was labelled (Do and Choi, 2007). Each biological replicate was labelled with Cy3 fluorescent dye (Amersham Mono-reactive Dye Pack, GE Healthcare), and with pooled common control aRNA products labelled with Cy5 (Amersham Mono-reactive Dye Pack, GE Healthcare). Unincorporated dye was then removed using the DyeEx 2.0 spin column purification kit (Qiagen). This was performed in accordance with a previously described protocol (Tacchi et al., 2011) for use in a two-colour microarray experiment.

2.7.2 Microarray hybridisation and scanning

Following Nanodrop spectrophotometry of samples to ensure adequate dye incorporation (Nanodrop ND1000, LabTech), 825 ng of each pooled Cy3- labelled experimental sample and 825 ng of Cy5-labelled common control were fragmented together using 11 µl of 10X blocking agent and 2.2 µl of 25X fragmentation buffer (Agilent) in 20 µl of Ultrapure RNase-free water (Invitrogen). Fragmentation was performed in the dark at 60 °C. 2X Hybridisation buffer prepared according to manufacturer's instruction (Aligent) was then combined with the solution in a 1:1 ratio and immediately applied to slides, taking care not to introduce bubbles. Approximately 103 µl of solution was applied to each microarray (16 arrays in total). Slides were then hybridised in a rotary-style hybridisation oven (Aligent) at 65 °C for 18 hours, appropriately balanced and rotation at 10 rpm. Slides used were 4x44K custom gene expression oligonucleotide Trout_imm_v1 microarrays, developed for rainbow Trout (Agilent design ID: 028918) (Castro et al., 2015), with a high number of immune related transcripts. Full details of the array platform are available at EBI array express (https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-3401/) under platform accession A-MEXP-2315.

After 18 hours, slides were removed from the oven and washed with Gene expression wash buffers 1 and 2 (Agilent) as per the manufacturer's instructions. Following airdrying in the dark, slides were scanned within an hour of washing. Scanning was performed using Gene-Pix Personal 4100A scanner (Axon Instruments), with adjusted PMT values to for a mean intensity ratio of equal Cy3:Cy5 signal, at a resolution of 5 μm. Initial analysis with Agilent Feature Extraction Software v9.5.3 (Aligent) identified array features and performed background correction of raw intensity values in generated *TIF. Files.

2.7.3 Data processing

Files obtained from slide scanning were read and processed using coding software R 3.5.0 (R Core Team, 2018), with use of the Limma and Bioconductor packages (Ritchie et al., 2015). This package allowed determination of mean feature intensity from data files and assignation of probe names. Average values were utilised for duplicate probes. Global loess normalisation was performed within arrays to limit intensity-dependant variation, and quantile normalization between arrays to limit experimental bias on results. Non-gene features, control spots, probes demonstrating even expression across treatments and RNA targets without data were then filtered from the dataset.

Use of the model matrix function for linear modelling allowed measurement of expression within groups (Cy3 labelled) relative to the background reference (Cy5 labelled). Groups could then be compared in a simple contrast matrix using the linear modelling and empirical Bayes methods for relative expression levels between different treatments (log fold change), with generation of Benjamin Hochberg (BH) adjusted p values to account for multiple comparisons (Benjamini and Hochbery, 2013; Smyth, 2004). Comparisons were for every combination of groups designated 'control', 'mild', 'moderate' and 'severe'.

Rainbow trout probes were mapped to human orthologs using an existing file that detailed best-matches of probe sequence to the human proteome using BlastX against the Ensembl database. All listed matches met the criteria of E-value < 0.001 using

BlastX. Probes that were not definitively matched to an associated HGNC (HUGO Gene Nomenclature Committee) gene symbols were excluded.

Chapter 3

3. Cnidarian jellyfish as vectors of bacterial pathogens of aquaculture

3.1 Overview

Whilst published research exists regarding cnidarian jellyfish as pest species of marine aquaculture, with their acute impacts well documented in farmed fish, less is known about subsequent consequences of exposure and sub-clinical impacts. Jellyfish are found in nearly every marine environment and until recently have been largely ignored in the context of aquaculture, but a growing field of research suggests their involvement in impaired finfish production. Research suggests the association of these jellyfish with secondary infections in fish following exposure, focusing on the bacterial pathogen *Tenacibaculum maritimum. T. maritimum* is, however, not the sole bacterial agent of gill disease in caged fish. Many bacteria are capable of infecting gills in the marine environment, the vector transmission of which by parasites and fish is well documented. Jellyfish might play host to other potential pathogens too, and so the aim of this chapter was to investigate the adherent microbial populations of medusozoan jellyfish species *Cyanea capillata, Neoturris pileata* and *Obelia geniculata* for other microbial pathogens. By identifying archived bacterial DNA to genus and then species level, potentially pathogenic bacteria were described.

Analysis of results of 16S sequencing identified a number of genera from jellyfish tissue, mainly from gammaproteobacterial taxa. Subsequent sequencing focused on species level identification of genera known to contain pathogenic species, namely *Aeromonas*, *Vibrio* and *Pseudomonas*. By targeting the *rpoD* gene in *Pseudomonas*, the intergenic spacer region (IGS) in *Vibrio* and the *GyrB* gene in *Aeromonas*, species level resolution was achieved for the majority of targeted isolates. From these, bacteria with interesting function and potential advantageous effect in the jellyfish lifecycle were identified, as well as potential pathogens of commercial aquaculture, including *Aeromonas salmonicida, Vibrio splendidus* and *Vibrio alginolyticus*. Isolation of these bacteria with pathogenic potential within the jellyfish microbiome has implications for the aquaculture industry, not just for the health of Atlantic salmon, but also the other

commercially associated species, including the cleaner fish utilised in biological parasite control.

3.2 Introduction

3.2.1 Cnidarian taxonomy and anatomy

The phyla of Cnidaria ('true' jellyfish) and Ctenophora (comb jellies) contain within them the gelatinous organisms commonly known as jellyfish (Mills, 1995). Members of these phyla are described together as jellyfish because of their shared observable anatomy; a characteristic gelatinous structure lacking complex digestive or circulatory systems, possessing only a simple diffuse nervous system (Brusca and Brusca, 2003). There exist 10,000+ species of Cnidaria alone, with varied morphology, however the stereotypical appearance of jellyfish is that of the medusa life stage of Scyphozoa, an upturned bowl with trailing tentacles (Zhang, 2011).

There are however important structural differences between Cnidaria and Ctenophora. The characteristic features of Ctenophora is their use of large, fused cilia for locomotion and possession of cells called colloblasts to adhere to prey tissue (Larink and Westheide, 2011). So, whilst Ctenophora are a distinct ecological concern due to their domination in areas such as the Adriatic Sea, they are not considered harmful to fish stocks, Ctenophora do not sting. Cnidaria on the other hand do sting. They possess specialised cells called cnidocytes, that can contain organelles called nematocysts, used in defence and for capture of prey (Springer and Holley, 2013). These cnidocytes are the stinging components of jellyfish and can be of concern to humans and aquatic organisms. Cnidarian nematocytes contain a collagenous barb and venom that is extruded on discharge of the sting (Springer and Holley, 2013). Nematocysts are found primarily on the tentacles and oral arms (Helmholtz et al., 2010), but can be present in all cnidarian jellyfish epidermal tissue (Springer and Holley, 2013). Discharge of the barb can puncture the tissue of prey (Jouiaei et al., 2015a), injecting venom. Species, geographical location and even age of the medusa appears to impact venom composition (Helmholtz et al., 2010; Helmholz et al., 2007; Mariottini and Pane, 2010; Purcell and Arai, 2001; Radwan et al., 2001). Antigenic, allergic and innate immune reactions can result from envenomation of humans, as a combined response to both the collagenous barb and envenomated toxins (Tibballs et al., 2011). Venom is thought to be composed of multiple, mainly proteinaceous, constituents, such as porins, neurotoxic

peptides and bioactive lipids (Nagai et al., 2000; Purcell, 1984). Little is understood about the toxic components of cnidarian jellyfish venom, an area of research with potentially fascinating implications not only in medical treatment and disease prevention in fish, but also for understanding the symbiotic relationship of cnidarians and their microbiota, and pharmaceutical discovery (Ovchinnikova et al., 2006).

It is the unique properties of cnidarian jellyfish that have allowed them to successfully colonise the oceans, with global geographical distribution, from polar to tropical waters (Lucas et al., 2014). The basic anatomy of Cnidaria is of two epithelial layers, epidermal outer and endodermal inner surfaces separated by an extracellular matrix (Brusca and Brusca, 2003; Larink and Westheide, 2011). The gelatinous mesoglea of which jellyfish are mainly composed is of low cellularity overall and metabolically inexpensive to produce. Containing mainly water, collagen, salts and few cell types, jellyfish body composition is approximately 95% water overall (Johnsen, 2000). Due to this, jellyfish have relatively low metabolic demand. This allows them to grow as much as 2.2x faster than other pelagic organisms, even when comparisons are corrected for carbon composition (Josephson, 2004; Neubauer, 2012; Pitt et al., 2013), leading to population booms.

The basic life stages of most jellyfish are the medusa free-swimming stage, and the benthic polyp stage. Polyps grow on various marine substrates and appear more like gelatinous corals or macroalgae (seaweed) (Fautin, 2002). Medusa reproduce sexually to create more medusa, and polyps asexually bud to form either medusa or more polyps (Brusca and Brusca, 2003). Optimal conditions/triggers for medusa production are not fully understood in most species, but temperature, light, salinity and availability of food appear to influence the production of medusa. There seems to be variation in the optimal values of these factors between species (Arai, 1997; Boero et al., 2008; Fautin, 2002; Stenseth et al., 2004). When medusal production does occur however, it can often occur on a large scale and result in aggregations of jellyfish known as blooms (Mariottini et al., 2008)

3.2.2 Jellyfish incidence and reporting

As well as Scyphozoa ('true' jellyfish) (Springer and Holley, 2013), there exist a number of other described classes within the Cnidarian phylum. These include Anthozoa (including corals), Cubozoa, Polypodiozoa (parasites) and Hydrozoa (hydroid jellyfish). The most common species of jellyfish in Scottish waters are, in order of most reported sightings by the public; *Aurelia aurita* ('moon jellyfish'), *Chrysaora hysoscella* ('compass jellyfish'), *Cyanea capillata (*'lion's mane'), *Cyanea lamarckii* ('blue jellyfish') and *Rhizostoma pulmo* ('barrel' or 'cauliflower jellyfish'). These are all Scyphozoan species native to UK waters (Pikesley et al., 2014). These data on abundance were collected as reports of sightings and standings of adult medusa by the public as part of the 2014 Marine Conservation Society Jellyfish Survey, which also provided sighting reports of Scyphomedusae *Pelagia noctiluca* 'Mauve Stinger' and two Hydrozoa, *Physalia physalis* 'Portuguese Man-of-War' and *Velella velella* 'By-the-wind Sailor'. This survey did not however report sightings of the smaller, harder to detect jellyfish within the class Hydrozoa. These jellyfish can be difficult to observe due to their small size and are often overlooked as a result (Kintner and Brierley, 2018). A huge variety of hydromedusa species are however thought to be native to Scottish waters, examples of which include *Muggiaea atlantica*, *Lizzia blondina, Phialella quadrata*, *Neoturris pileata* and *Obelia* species (Kintner et al., 2013; Larink and Westheide, 2011). True incidence and population estimates are difficult to obtain for any jellyfish, but this is particularly true for microscopic species.

Despite the recent news reports of increasing incidence of jellyfish blooms, scientific evidence is as yet unclear whether incidence of large jellyfish blooms are increasing globally (Sanz-Mart ın et al., 2016). A lack of quantitative historical jellyfish population data makes differentiating seasonal shifts and short term eco system fluctuations from long term population trends difficult (Mills, 2001). Increasing evidence of blooms may be due only to improved reporting, with increasing mortality events in aquaculture attributable to the fact aquaculture itself is increasing. Blooms are a feature of jellyfish life cycles, with rapid population explosions during favourable conditions made possible by their short life cycle, rapid growth and asexual reproduction (Mills, 2001).

Most lifecycles are annual, with favourable conditions occurring seasonally, influenced by local and ocean-wide factors, and requiring specific conditions for different stages of the life cycle. What is known is that our changing climate is altering ocean conditions, with an increase of 0.31°C in the top 300 m of the world's oceans in the last sixty years (Levitus et al., 2000). These warming ocean temperatures are predicted to predispose to increased jellyfish bloom incidence (Lynam, Lilley et al., 2011), although much is still to be learnt regarding the dynamics of jellyfish domination (Purcell, 2005). *Pelagia noctiluca* for example favours marine temperatures exceeding 10°C in winter and less than 27°C in summer, with salinities of 35–38 (Goy et al., 1989; Purcell et al., 1999). Since winter warming of the oceans is advantageous for the survival of this predominantly warm water species, it might reasonably be assumed that populations are going to increase in the warming North East Atlantic (Doyle et al., 2008). Anthropogenic effect on the pelagic environment can also have consequences for jellyfish populations. Eutrophication can favour jellyfish (Arai, 2001), as can the presence of floating farm architecture that acts as substrate for polyp attachment (Guenther et al., 2010). Species with benthic polyp stages of production in particular can therefore benefit from the presence of aquaculture-introduced local factors (Doyle et al., 2008).

Jellyfish cannot be observed via satellite imagery, unlike chlorophyll containing phytoplankton, due to their opaque gelatinous bodies (Johnsen and Widder, 1999, 1998), and so no early warning system exists for offshore jellyfish blooms. No routine monitoring is conducted either by aquaculture industries for jellyfish around fish farms. Although monitoring techniques are similar to those for phytoplankton, conducted using zooplankton nets and microscopy (Arai, 1997), sampling for jellyfish is rarely performed by aquaculture personnel. Any observation or documentation of jellyfish in the water then is usually as an incidental, or through specific investigation following an outbreak of unexplained gill disease. The sporadic occurrence and presence throughout the water column of jellyfish make large-scale assessments costly to conduct, and little data are available regarding coastal incidence and intensity of benthic populations. Hydromedusa have been recorded as one of the most diverse small gelatinous zooplankton around the coast of Southern Ireland (Baxter et al., 2012a), but information

is lacking in the population estimates of these organisms. Coastal and open water environments host different species of jellyfish, meaning abundance estimates of open coastal surveys likely cannot be extrapolated for population prediction of species in coastal locations (Goy, 1991), which is where mariculture is most likely to be located. Considering abundance can differ drastically though with relatively little change in distance or even time, a multitude of factors compound the difficulty in observing and predicting cnidarian populations.

3.2.3 Jellyfish of concern and the risk to aquaculture

Until recently, little was known about the impact of jellyfish on aquaculture, but it is now well accepted that blooms of a number of species can lead to large scale mortality events of farmed fish. Blooms of *Aurelia aurita, Pelagia noctiluca, Phialella quadrata* and *Solmarisidae* have all been implicated in fish kills in a variety of aquaculture species around the world (Bosch-Belmar et al., 2017a, 2016b; Bruno and Ellis, 1985; Forseth et al., 2017; Mitchell et al., 2011b; Munro, 2014; Raffaele, 2013). Lack of routine monitoring of jellyfish populations currently inhibits the study of the risk of cnidarian jellyfish to aquaculture. However, a number of studies have explored the consequences in fish of non-lethal jellyfish exposure, and an increasing number of publications suggest that, in addition to mass mortalities associated with jellyfish blooms, sub-lethal pathology and subsequent bacterial disease are an important consequence of jellyfish exposure.

Both free-swimming and sessile polyp life-stages have been proven to pose a risk to aquaculture, with impact ranging from the acute to potentially sub-clinical. Free swimming Hydrozoa and some Scyphozoa are small enough to pass through the nets of sea cages and to be inhaled by fish (Mitchell et al., 2011b), stinging the epithelial tissue of gills and the GIT (M. D. Powell et al., 2018). Larger Scyphozoa are easily damaged in strong currents or bad weather, so pieces of tissue can pass through nets and sting fish (Baxter et al., 2011a, 2011b; Rodger et al., 2010). Additionally, sessile polyp life stages can also impact fish health. A thick biofilm on nets can reduce water flow and oxygen levels, so net washing is performed by inshore Atlantic salmon producers to clean nets (Baxter et al., 2012b; Hodson et al., 1997). Unfortunately, power washing

nets can dislodge cnidarian polyps and allow their dispersal throughout the water column, leading to stinging of the fish contained therein (Fitridge et al., 2012; Guenther et al., 2010; Hodson et al., 1997).

Exposure of fish to individual *C. capillata* medusae has been demonstrated to illicit a clear behavioural and physiological response indicative of stress, alongside observable skin and gill lesions (M. D. Powell et al., 2018). *A. aurita* and *P. noctiluca* too have been experimentally demonstrated to impact fish health (Bruno and Ellis, 1985; Mitchell et al., 2011b; Raffaele, 2013). Even tiny hydrozoan jellyfish species, the populations of which cannot be detected with the naked eye and so go largely unreported, apparently have a role in increased salmonid mortalities. Species such as *Phialella quadrata* and *Solmarisidae* (Munro, 2014), *Ectopleura larynx* (Baxter et al., 2012b), *Obelia* sp. and *Lizzia blondina* (Kintner and Brierley, 2018), have all been implicated in negative impact on fish health. The impact of cnidarians then clearly extends beyond obvious large bloom events, with an expanding field of research indicating that lower density populations of jellyfish and potentially as yet unexplored species might also be of importance to aquaculture (Mitchell et al., 2012).

3.2.4 Known impacts of jellyfish on fish gills

Gill pathology in fish following exposure to a number of cnidarian species has been described (Bosch-Belmar et al., 2016b; Mitchell et al., 2011b; M. D. Powell et al., 2018), as well as the toxicological symptoms associated with envenomation (Baxter et al., 2011a; Mitchell et al., 2012; Rodger et al., 2010). Direct mechanisms of impact are though still poorly understood. Mortalities of fish are thought to occur during a jellyfish bloom not only because the gelatinous structure of these organisms can impair oxygen exchange by obstructing the gills, but also because they sting the fish (Mitchell et al., 2011a). Gill epithelium in particular is very delicate and is thought to be damaged by physical stinging (Baxter et al., 2011a; Mitchell et al., 2012; Rodger et al., 2010). Nematocyst tubule length and tissue composition appear to determine how deeply the discharged barb will penetrate, and venom composition its effect. Some stings can reach the capillary network, leading to more generalised systemic reactions, such as in the serious stings delivered by Cubomedusae. Death is not an uncommon outcome of

Cubomedusae envenomation in humans*; Chironex fleckeri* venom can cause cardiopulmonary arrest within minutes (Burke, 2002). Stings by *P. noctiluca* are documented as causing painful wheals and pruritus, and *C. capillata* also delivers a painful sting. Species known to illicit painful stings in humans are documented as negatively impacting fish, but so too are jellyfish considered non-stinging in humans (Helmholz et al., 2010; Mitchell et al., 2011b; M. D. Powell et al., 2018). This is likely since salmon gills lack the protective, stratified squamous layers of human epithelium.

Previous research has explored the histological consequences of jellyfish exposure in gills (Mitchell et al., 2011a; M. D. Powell et al., 2018). Following exposure to *A. aurita*, an initial acute reaction involving haemorrhage, necrosis and epithelial sloughing occurs, progressing to an obvious inflammatory granulocyte response and oedema. At peak pathology (48 hours post exposure) large areas of damage and lysis of erythrocytes can be observed in Atlantic salmon gill tissue (Baxter et al., 2011b). Eventual pathology from *A. aurita* manifests as lamellar fusion and hyperplasia, with evidence of repair by 3 weeks post exposure. Exposure to *C. capillata* appears to elicit a similar response in salmonid gills, with an acute presentation of epithelial separation and oedema, focal haemorrhage and thrombus formation, progressing to inflammatory epithelial hyperplasia with thrombi visible within hyperplastic lamellae, resolving after approximately 28 days (M. D. Powell et al., 2018). Hydrozoan species *Phialella quadrata, Solmaris corona* and *Muggiaea atlantica* have also been linked too to sequential gill pathology in the marine environment (Baxter et al., 2011a), with mild gill disease observed in farmed fish following suggested high densities of these organisms. The specific cumulative impact of these organisms on salmonid aquaculture however is yet to be studied. There exists no information regarding the mechanisms of immune response to jellyfish in fish, and the impact of 'intensity' of exposure (number cnidarians /L) as well as long-term down-stream consequence of exposure on fish health are as yet unknown.

Although little is known about low level exposure of harmful cnidarian species or consequences of sub-acute trauma in farmed fish, one of the most commonly reported outcomes of jellyfish gill trauma is apparent bacterial colonisation of gills. Bacteria

observable in histological sections of damaged gill tissue and clinical bacterial disease are often reported subsequent to exposure (Baxter et al., 2011b; Rodger et al., 2010). This is hardly surprising, as infections are often facilitated by disarrangement of structural and immunological defence mechanisms by traumatic events. Subsequent colonization by bacterial agents capable of initiating disease-type symptoms in a host ('potential pathogens') is then possible from a variety of sources. Bacteria may come directly from overgrowth of microbial populations already present on the tissue, from opportunistic infection by bacteria present within the water column in the marine environment, or directly by vector transmission.

A wealth of literature exists regarding marine vertebrates and invertebrates, such as wild fish and parasites such as *L. salmonis* (sea lice), acting as carriers of bacterial diseases of salmonids (Barker et al., 2009; Belkin et al., 2005; Cusack and Cone, 1986; Nylund et al., 1991). A number of recent studies have considered the potential for jellyfish to act as agents too of transmission. For example, the jellyfish *P. quadrata* was implicated as a vector of the bacterium *Tenacibaculum maritimum* after exposure of caged Atlantic salmon to a large bloom of these jellyfish was followed by an outbreak of Tenacibaculosis (H. W. Ferguson et al., 2010). *T. maritimum* has been described as part of the microbiota of *Pelagia noctiluca* medusae as well (Delannoy et al., 2011), implying that jellyfish might host agents of bacterial disease within their microbiome as resident communities.

Current thinking of health professionals and veterinarians within the Atlantic salmon aquaculture industry is that unnoticed jellyfish exposure is to blame, or at the very least is exacerbating, unexplained gill disease in farmed stocks (pers. comm's, Scottish Sea Farms). An important component of this impact may be through the vector transmission of harmful bacteria. Jellyfish trauma certainly disrupts gill tissue, with a presumptive impact of impaired defence against microbial colonisation, either from environmental populations or the cnidarians themselves. Through current underreporting and lack of routine sampling for jellyfish populations, important predisposing factors to complex gill pathologies remain to be understood. Bacteria are after-all nothing if not opportunists, exploiting any available ecological niche to survive and proliferate.

3.2.5 Jellyfish microbial communities

3.2.5.1 Existing knowledge

Despite their relatively uncomplicated immune system, lacking a physical barrier between epithelium and microbes and with no adaptive immune system or phagocytic cells, Cnidarian hosts are still able to modulate their microbiome. The surface of cnidarian tissue is coated in a protein and lipid mucus layer (Ducklow and Mitchell, 1979), which provides an energy rich substrate for bacteria (Ducklow and Mitchell, 1979). The microbiota residing in this nutritionally advantageous environment are thought to be less diverse than those of vertebrates (Fraune and Bosch, 2007), but still specialized and compositionally distinct from environmental populations (Cleary et al., 2016; Weiland-Brauer et al., 2015). Medusozoan jellyfish have also been demonstrated to produce a variety of antimicrobial peptides for modulation of their adherent bacterial population (Bhosale et al., 2002; Ovchinnikova et al., 2006; Tinta et al., 2012).

Different cnidarian species appear to harbour significantly different adherent bacterial populations (Weiland-Brauer et al., 2015). Research of the cnidarian microbiome emphasises the presence of a core microbiota, prevalent in all individuals of a species, alongside additional communities that vary by individual in their relative composition, occurrence and abundance (Brown et al., 2017; Ursell et al., 2012; Webster et al., 2018). Association of sponges and their microbial communities is considered to be one of the most diverse and complex marine symbiotic relationships (Moran and Sloan, 2015; Pita et al., 2018; Rohwer et al., 2002). The adherent microbiota of medusozoan species do appear though to vary with life stage (Weiland-Brauer et al., 2015) and geographical location (Hao et al., 2015; Schmitt et al., 2012). Some bacterial isolates appear to reside within the tentacle tissue itself (Schuett and Doepke, 2010), with a suggested role for these endobiotic microbes in toxin production, such as occurs through the association of host and bacteria in some other marine species (Haygood et al., 1999; Lee et al., 2000). The potential for symbiont roles of bacteria in the lifecycle of these organisms is also being explored (Weiland-Brauer et al., 2015). The majority of microbial research in cnidarians is currently focused on the discovery of antimicrobial peptides, for example

the discovery of a protein known only as CAP-1 from the Gammaproteobacteria *Pseudomonas* present in the tentacle microbiome *C. capillata* (Yin et al., 2016).

3.2.5.2 Investigating the microbial communities of Cnidaria

Investigations of microbiota can focus on overall microbial communities, to understand microbial relationships and identify dominant phyla, or isolation of a specific pathogen, such as in a disease diagnosis situation. A variety of techniques exist that might be appropriate for the objective of species-level identification of potential pathogens within mixed microbial populations, all with different advantageous and disadvantages and appropriate applications depending on the sample material and study objective.

Many studies exist that utilize 16S alone for genus and even species level identification. Modern research however emphasizes the need for additional techniques to unambiguously identify bacterial isolates to species level. Alternative sequencing can be conducted, such as using species-specific primers, or more general sequencing of housekeeping genes. Species-specific sequencing is a method particularly of use in disease diagnosis or isolation of bacteria from sterile body cavities, whereas housekeeping genes are present in all species within a genus and more appropriate for polymicrobial isolates or identification of unknown components of communities.

3.2.6 Jellyfish as hosts of potential pathogens

This chapter of work sought to investigate the microbiome of jellyfish for the demonstration of bacteria associated with disease in aquaculture that might be transmitted. Samples of bacterial genomic material were obtained from previous PhD student Anna Kintner (AK) as unresolved work from a project that investigated the occurrence and impact of jellyfish blooms on salmon aquaculture. Samples of jellyfish tissue were collected from the coast of Shetland by AK and progressively sub-cultured to obtain single populations of bacteria before DNA extractions were performed. AK's work had aimed to identify the marine pathogen *Tenacibaculum maritimum* using a

culture technique, and although no *T. maritimum* were identified, archived material remained to be classified.

The work of this chapter was therefore performed to taxonomically classify isolates obtained from the work of AK, with a focus on identification of any pathogenic isolates. Bacterial samples were obtained from the adherent microbiota of three cnidarian jellyfish (*Cyanea capillata*, *Neoturris pileata* and *Obelia geniculata*) that might impact salmonid aquaculture. First, 16S gene sequences were analysed to identify archived monoculture DNA to genus level. Appraisal of additional selected sequencing when allowed classification of isolates to species level within the genera associated with fish disease. Although, through inclusion of a culture step, conclusions cannot be drawn regarding microbial diversity of the microbiomes of these jellyfish, a number of potentially pathogenic bacteria were identified with the genera V*ibrio*, *Pseudomonas* and *Aeromonas*. Results of this study therefore support existing research regarding the potential of jellyfish to act as vectors of bacterial disease, as well as identifying microbes with potential involvement in the cnidarian lifecycle. Overall, identification of novel pathogenic microbes as part of the microbial community of Cnidaria demonstrates the ability of these apparently common jellyfish to host and transmit multiple bacteria harmful to salmonid aquaculture, a finding with potentially global implications.

3.3 Methods

This laboratory work was conducted using archived genomic material from previous PhD student AK, University of St Andrews. Some techniques conducted solely by AK have been included and clearly highlighted to give clarity to the source and methodology of previous work.

3.3.1 Sample Material

Jellyfish isolates were obtained from four locations around the coast of Shetland: Lunna 60.419004, -1.105436, Redayre 60.193509, -1.405769, Roe Sound 60.377864, -1.384974 and North of Papa 60.130489, -1.342794 by AK in September 2012. Three jellyfish species were collected, *Cyanea capillata* (n=8), *Neoturris pileata* (n=7) and *Obelia geniculata* (n=10), with species identification based on morphological characteristics. Each specimen was rinsed with sterile sea water to remove loosely associated environmental microorganisms before being suspended overnight in a purpose-built sterile tank facility. UV-treated seawater at 11°C and a median salinity of 33 passed in a continuous flow over intact cnidarian specimens. Concurrently collected sea water samples were also obtained from each location into 1L sterile containers.

Samples were then used by AK for bacterial culture. Initial broth cultures were conducted using sections from all jellyfish species collected. Samples were obtained from *C. capillata* by removal of small 1cm^2 sections of fishing tentacles and by sterile swabbing of the gut. Smaller *N. pileata* and *O. geniculata* (0.5-10mm and 8-11mm in length respectively) were cut in half and used directly in broth culture. Two media, selected by AK to encourage growth of pathogenic bacteria, were utilized for each sample - Bovine Brain-Heart Infusion (BHI) and *Flexibacter maritim*us Media (FMM) (Pazos et al., 1993; Rosenow, 1919). Each sample was incubated for 24 hours with occasional agitation at 25°C to encourage optimal growth of bacteria within, then each sample was streaked onto BHI and FMM agar plates using a sterile inoculation loop. Plates were incubated for a further 24 hours at 25°C. Resulting polycultures were divided for further plate culture until monocultures, as judged by morphological appearance, were achieved. Colonies

from these plates were then selected and cultured in corresponding broth media for amplification of bacterial quantities over a further 72 hours at 25°C. Samples of sea water obtained from sampling visits were also utilized in broth culture. Following settling, 0.5mL samples were obtained from the bottom of containers and placed in separate broth media for incubation alongside cnidarian samples. 0.5mL samples were also collected from the tank systems and cultured in-kind. Culture conditions were selected by AK to optimise the growth of the maximum possible pathogenic microbes (Starliper, 2013).

DNA was extracted by AK using a crude boiling approach with 1 mL from each broth culture first being centrifuged and the supernatant discarded. The pellet was re-suspended in 0.5 mL tris-EDTA (TE) buffer and heated at 95˚C for 10 minutes. Samples were centrifuged again and stored at –80˚C. The preceding work was conducted independently from the work of this study by AK between 2012-2014.

3.3.2 Polymerase Chain Reaction and sequencing

Amplification by polymerase chain reaction was conducted using universal primers as described in Chapter 2 (Section 2.4.1) and sequenced in a single direction to obtain an approximately 800bp fragment of the 16S gene from each bacterial monoculture. Resulting nucleotide sequences were edited using Geneious software (https://www.geneious.com) (Kearse et al., 2012) prior to comparison against the Genbank database using BlastN. Nucleotide to nucleotide comparisons of sequences obtained against Genbank submissions generated lists of matches that were filtered based on the parameters of 100% coverage and over 97% identity. Additional comparisons were also performed against the curated database 'Ribosomal Database Project' RRID:SCR_006633 (Cole et al., 2014).

Following genus identification from the 16S sequences, further gene loci were targeted to enable species-level identification of a subset of samples from genera *Pseudomonas, Vibrio* and *Aeromonas.* This was conducted using primers previously utilized in the literature for resolution of species to amplify house-keeping genes within these genera, as detailed in Chapter 2 (Section 2.4.1). Briefly, the *GyrB* gene was sequenced for

resolution of *Aeromonas* isolates, as well as the *rpoD* gene from *Pseudomonas* and Intergenic-spacer region (IGS) from *Vibrio*. Following clean-up (Section 2.5), *Pseudomonas* and *Aeromonas* PCR products could be directly sequenced (Section 2.4.1). PCR of the IGS region of monocultures has however been demonstrated to yield variable base-pair sequence amplicons (Gurtler and Stanisich, 1996), and so sub-cloning was performed using the products of PCR in *Vibrio*. Individualised fragments were obtained by colony picking (4 – 9 from each *Vibrio* sample) and sequenced.

3.3.3 Computational analysis and figure construction

Following required editing of sequences using Geneious 9.0.5 software (https://www.geneious.com) (Kearse et al., 2012), sequences were compared nucleotide to nucleotide using BlastN against the NCBI Genbank database. Matches meeting the required parameters were those with 100% coverage and 97% identity or higher for taxonomic classification. Additional comparisons were made where relevant against curated databases, including the Ribosomal Database Project RRID:SCR_006633 (Cole et al., 2014) for results of 16S sequencing and the PseudoMLSA Database (Bennasar et al., 2010) now found at http://microbiologia.uib.es/bioinformatica). Multiple sequence alignments were produced with JalView (Waterhouse et al., 2009) using alignments generated by use of the default settings of CLUSTAL omega (Sievers et al., 2011). Phylogenetic tree construction was performed using CLUSTAL omega derived alignments and the software program MEGA 7.0 (S. Kumar et al., 2016). Trees were, unless otherwise stated, constructed using the neighbour-joining method with maximum likelihood distances, 1500 replications and with bootstrap values over 97 illustrated. Trees were rooted to outgroups composed of closely related taxa out with the species of interest.

3.4 Results

3.4.1 Genus level classification of archived bacterial monocultures

Bacterial isolates were obtained from jellyfish *C. capillata* (n=8), *N. pileata* (n=7) and *O. geniculata* (n=10). Initial 16S sequencing of bacterial monoculture derived colonies yielded 204 sequences in total from jellyfish sampled (n=25). A total of 67 sequences were obtained from the species *C. capillata*, 52 from *N. pileata* and 85 from *O. geniculata* individuals. Several sequences were first removed from the analysis as in-source duplicates, identical across their length to another sequence from the same source. Sequences determined not to be true monocultures, from the confused or duplicate chromatograms obtained during sequencing, were also removed from the analysis. Sequences considered to be of environmental origin were also removed. These were cnidarian derived sequences with 100% identity with environmental or aquarium derived cultures. Following primer removal and conservative trimming a number of additional sequences were also excluded from the analysis – those determined to be poor quality sequences, based on chromatograms, and any sequences less than 400 bp in length. A total of 57 unique sequences remained for analysis, of average sequence length 573 bp.

Based on 16S sequencing it was not possible to classify all isolates to genus level using BLASTN against the NCBI database. Nevertheless, genus level identification was possible for 42 sequences in total (**Table 3.1, Figure 3.1**). Initial 16S sequencing results were dominated by the phylum Proteobacteria, with low numbers of Firmicutes and Actinobacteria. Twelve distinct genera were identified overall from the samples obtained from the three species of jellyfish. They constituted nine genera from *C. capillata*, three genera from *O. geniculata* and six from *N. pileata*. Some of these sequences included matches to multiple genera and, after excluding mismatches due to misclassifications or use of historical nomenclature within the database, several could still only be classified to a higher taxonomic level.

Aeromonas were obtained solely from *C. capillata* jellyfish, from both the tentacle and gut tissue. Single *Shewanella* and *Bacillus* isolates were obtained from *C. capillata*

tentacle. Isolates obtained from *N. pileata* were exclusively *Vibrio, Pseudomonas* and *Pseudoalteromonas*. All *Psychrobacillus* isolates were obtained solely from *O. geniculata*.

Table 3.1 Jellyfish associated genera

Jellyfish species	Identified Bacterial Genera
C. capillata	*Shewanella, Vibrio, Morganella, Aeromonas, Pseudomonas, Bacillus, Acinetobacter, Exiguobacterium, Pseudoalteromonas*
N. pileata	*Vibrio, Pseudomonas, Pseudoalteromonas*
O. geniculata	*Arthrobacter, Vibrio, Psychrobacillus, Paenibacillus, Exiguobacterium, Pseudoalteromonas*

Table 3.1*: Genera identified following sequencing, filtering and classification against the Genbank database of jellyfish-derived bacterial 16S sequences. Isolates with matches to multiple genera within the Genbank database have been excluded.*

In this study, despite combination of both forward and reverse sequences obtained for *Pseudomonas* isolates, species level classification was still not possible for any of the *Pseudomonas* sequences in question, based on the standard parameters of 97% identity and 100% coverage against the Genbank database. Whilst resulting sequences did not overlap, indicating the entire gene sequence had not been obtained, an average total of 1200bp was compared against the NCBI Genbank database for these isolates. The available literature on *Pseudomonas* suggests that the 16S gene is nearly identical across all species, confirmed by construction of an alignment of 16S genes from *Pseudomonas* type strains (not shown). The decision was therefore made that further sequencing would focus on the use of additional alternative primers instead of additional 16S sequencing for species level resolution of isolates.

Figure 3.1 Sunburst to genus level

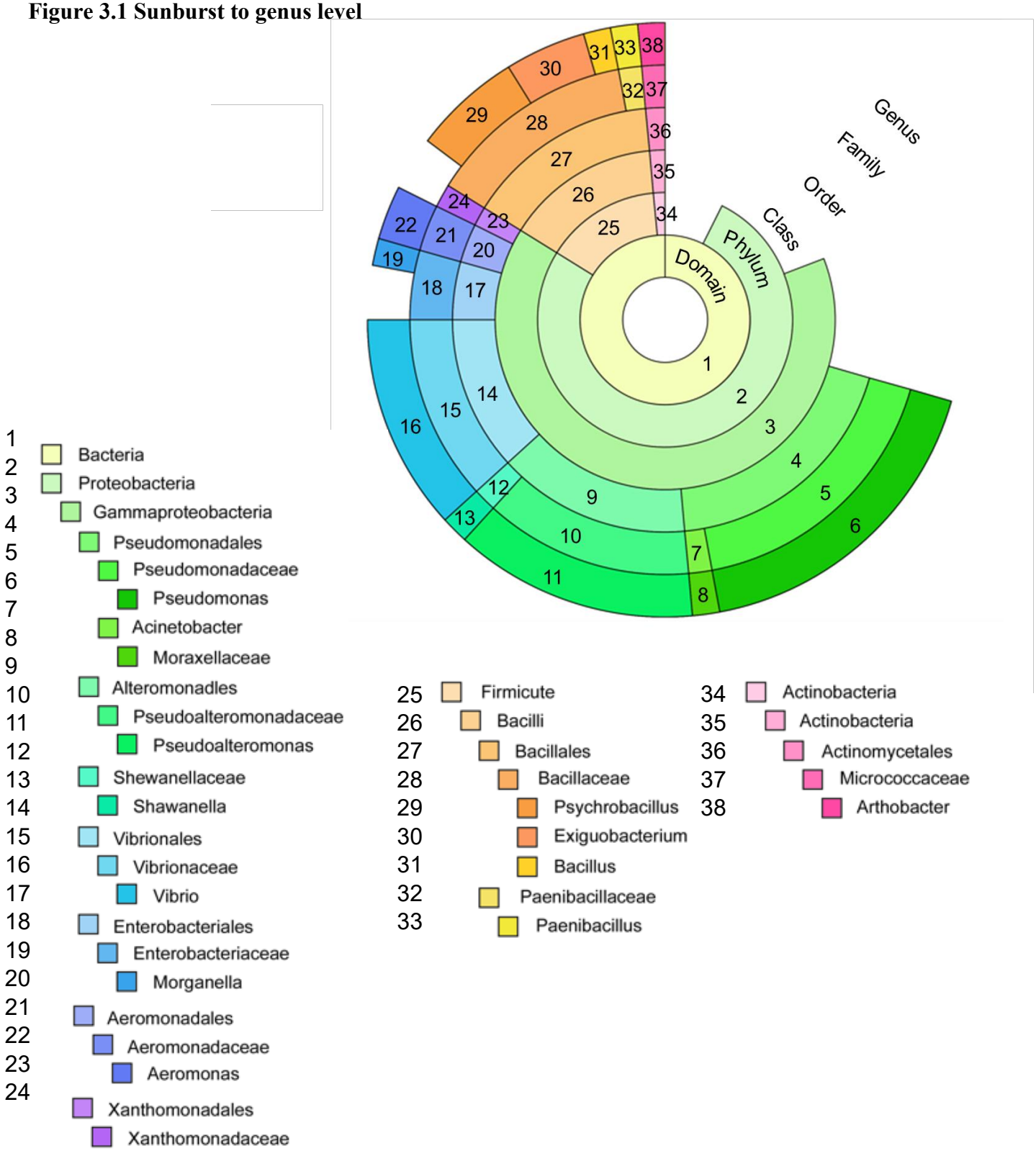


Figure 3.1*: Sunburst diagram illustrating the taxonomic classification results of 16S sequencing to genus level. Sequences were assigned using BLASTN against the Genbank database with a cut off of >97% identity and 100% coverage.*

3.4.2 *Aeromonas* sequencing

16S sequencing identified two potential *Aeromonas* isolates. One sample matched using BLASTN with only *Aeromonas* isolates within the NCBI Genbank database. The other *Aeromonas* isolate matched with two genera within the Genbank database. Matches were to several *Aeromonas* species and *Haemophilus piscium*, however the Genbank match *Haemophilus piscium* was shown to be historical nomenclature for the atypical *A. salmonicida* strain *Aeromonas salmonicida piscium*, and so could then also be classified as an *Aeromonas* isolate. Lack of sequence variation within the obtained amplicons and publicly available sequences precluded species level classification of these *Aeromonas* isolates based on 16S sequences alone. Sequencing primers to determine species level identification for the potentially pathogenic genera of interest were selected based on a literature review. Selection of the *GyrB* primer for *Aeromonas* was based on previous research regarding species level classification of *Aeromonas* isolates (Sen, 2005; Yáñez et al., 2003) . Results of this sequencing allowed classification of the two isolates to *Aeromonas molluscorum* and *A. salmonicida* based on their matches within the Genbank database and construction of a *GyrB* phylogenetic tree (**Figure 3.2**).

Multiple sub-types exist of the potential pathogen *Aeromonas salmonicida*. All cause furunculosis, with the majority causing 'atypical' forms of disease, and *A. salmonicida salmonicida* causing the 'typical' furunculosis. The gene encoding membrane protein *VapA* is a sequence proposed for distinguishing between *A. salmonicida* subtypes, however our attempts to sequencing this gene using recommended primers (Gulla et al., 2016) were unsuccessful in this study. Variation to the protocol in an attempt to achieve results using these primers universally failed.

Figure 3.2: Aeromonas GyrB phylogenetic tree

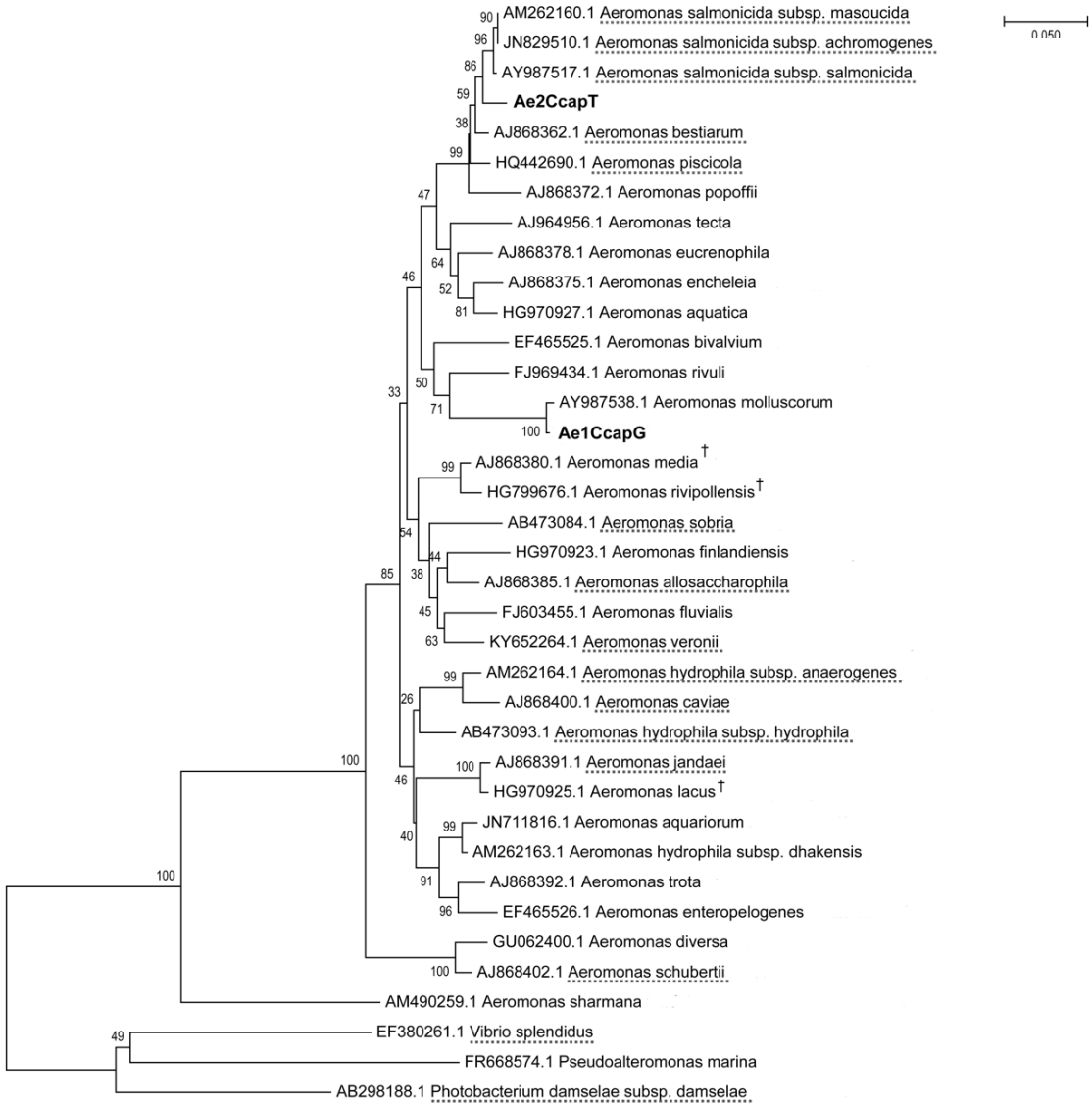


Figure 3.2: *Phylogenetic tree demonstrating relationship of study derived Aeromonas GyrB sequences and partial type strain sequences. Type strains were identified from the online resource http://www.straininfo.net. Tree was constructed as described in methods section using neighbour-joining method with maximum likelihood distances. Numbers represent bootstrap values for each node based on 1500 replications and the tree is rooted to outgroup Vibrio splendidus, Pseudoalteromonas marina and Photobacterium damselae. Sequences derived from this study are highlighted in bold. Species associated with fish disease are underlined (Beaz-Hidalgo et al., 2010; Godoy et al., 2010; Kozińska, 2007). Sequences marked with a dagger symbol † were obtained from NCBI taxonomic samples not confirmed as type strains. Sequence alignment for this tree is available in* ***Appendix A.1***

3.4.3 *Pseudomonas* sequencing

Eleven unique sequences were classified as belonging definitively to the genera *Pseudomonas* based on 16S sequencing. An additional four sequences were also included as suspected *Pseudomonas* (due to the presence of some *Pseudomonas* species within BLASTN matches for 16S sequences against the Genbank database). Sequencing of the *rpoD* gene identified six of these isolates to species level based on comparison using BLASTN against the Genbank database alone. Three isolates were confirmed as *Pseudomonas fulva*, and one as *Pseudomonas fluorescens*. Two were found to not belong to the genus *Pseudomonas*, but instead were confirmed by *rpoD* sequencing to be *Pseudoalteromonas aliena* and *Acinetobacter guillouiae*. Further phylogenetic analysis (**Figure 3.3**) suggests additional species level assignments for a further four samples as *Pseudomonas alkylphenolia* and *Pseudomonas guineae*, leaving four samples not classified to species level.

3.4.4 *Vibrio* sequencing

Species level resolution was achieved for all eight *Vibrio* samples by utilising primers designed to target the 16S-23S intergenic spacer region (IGS). Multiple genetically distinct IGS regions are obtained from single monoculture genomes, generating multiple sequences that might inform species classification for each Vibrio sample isolate. Within each sample, some of these sequences proved to be diagnostic to the species level and others did not, highlighting the need for sequencing multiple fragments. For classification of each sample to species level, a consensus classification was reached from appraisal of sequences that were considered diagnostic. IGS sequences were considered diagnostic if the BLASTN matches obtained had >97% identity and 100% coverage for a single Genbank submission. Those with multiple high identity matches or those that did not meet these parameters were considered insufficiently specific and non-diagnostic. The nucleotide sequence of resulting IGS sequences varied both within individual samples as well as from the closest matches within the Genbank database. An exemplar of this variation is demonstrated in the alignment for Sample A (**Figure 3.4)** Alignments for remaining samples (B-H) are also provided (**Appendix A.3**). This variation in IGS

amplicon length appears to be partially due to the presence of tRNA genes that vary in number and type across the multiple spacers present (Gurtler and Stanisich, 1996; Kong et al., 1999). In addition to amplification of the IGS region, the primer set utilised also amplified a similar length region of a DNA helicase-encoding gene from Vibrio Samples C and D, the results of which are excluded from alignments and subsequent analysis. Sequence matches for Vibrio samples are summarized in **Table 3.2**, with full details of all BLAST matches for all sequences obtained from the IGS region also available (**Appendix A.4**). An average of four IGS amplicons were obtained for each sample, some of which could be considered diagnostic and some of which could not. Diagnostic sequences were then utilized to reach consensus classifications for all *Vibrio* isolates.

Ultimately, three species of *Vibrio* were definitively identified: *Vibrio splendidus, Vibrio crassostreae* and *Vibrio alginolyticus*. Two potentially novel isolates were also identified, based on their failure to adequately match available species within the Genbank database. *Vibrio splendidus* was obtained from *O. geniculata, N. pileata* and *C. capillata* gut tissue. *Vibrio crassostreae* was isolated from *O. geniculata* tissue and *Vibrio alginolyticus* was isolated from *N. pileata.* The two potentially novel *Vibrio* isolates generated different IGS sequences to each other and can be considered different species. These isolates potentially represent new species or strains, as none of the obtained IGS fragment sequences could be matched with >97% identity to NCBI Genbank database submissions. The closest Genbank matches to these novel sequences were *V. alginolyticus* for sample F, with the closest sequence demonstrating 96% identity to available *V. alginolyticus* data within Genbank, and *V. splendidus* for sample E, with 95% identity for the closest Genbank match. The sequences from these novel *Vibrio* species/strains were deposited in Genbank with accession numbers MH310872 to MH310876.

All samples sequenced for species classification and accession numbers are summarized in **Table 3.2**, including metadata regarding sample source, final species designation and corresponding accession numbers. 16S sequences were deposited as accession

MH205967-MH205988 and results of further sequencing as MH310838- MH310882 within the NCBI Genbank database.

Figure 3.3 Pseudomonas rpoD phylogenetic tree

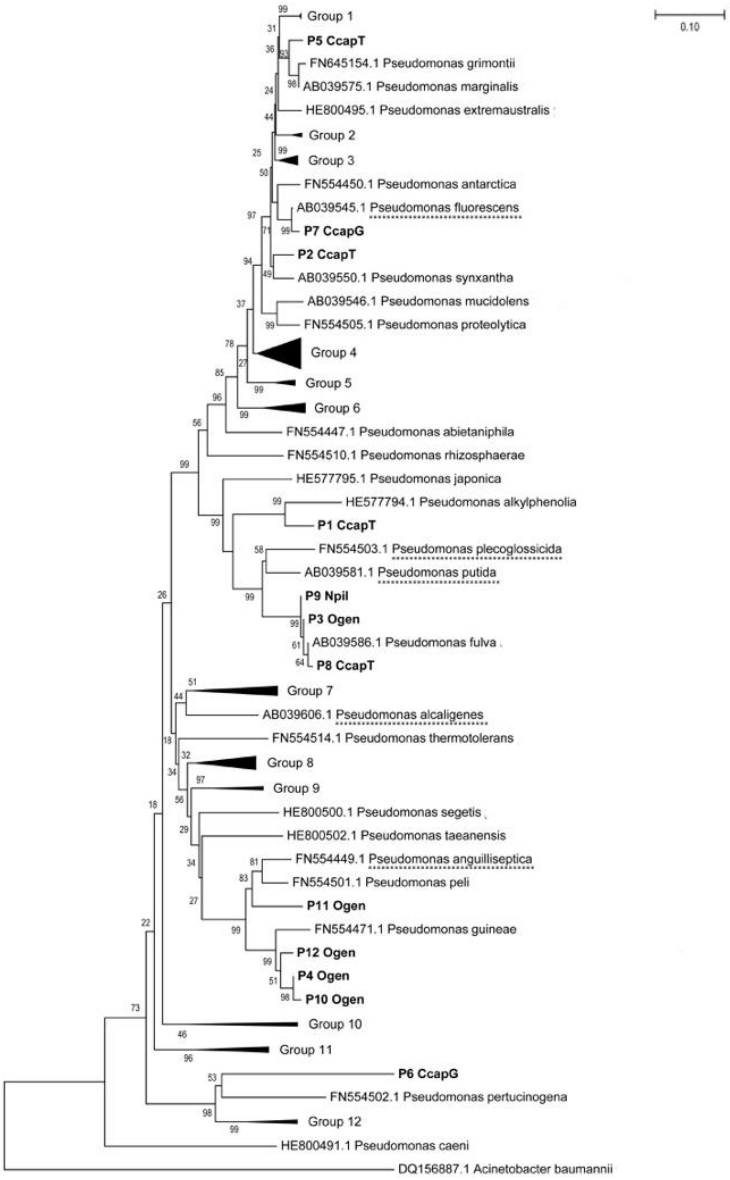


Figure 3.3: *Phylogenetic tree demonstrating relationship of study derived rpoD sequences and Pseudomonas sequences obtained from PseudoMLSA curated list of type strains. Tree was constructed as described in methods section using neighbour-joining method. Numbers represent bootstrap support values for each node based on 1500 replications and tree is rooted with Acinetobacter baumannii. Sequences required from this study are highlighted in bold. Sequences associated with fish disease are underlined. Sequence alignment in appendix A.2.*

Figure 3.4 Vibrio IGS alignment, sample A

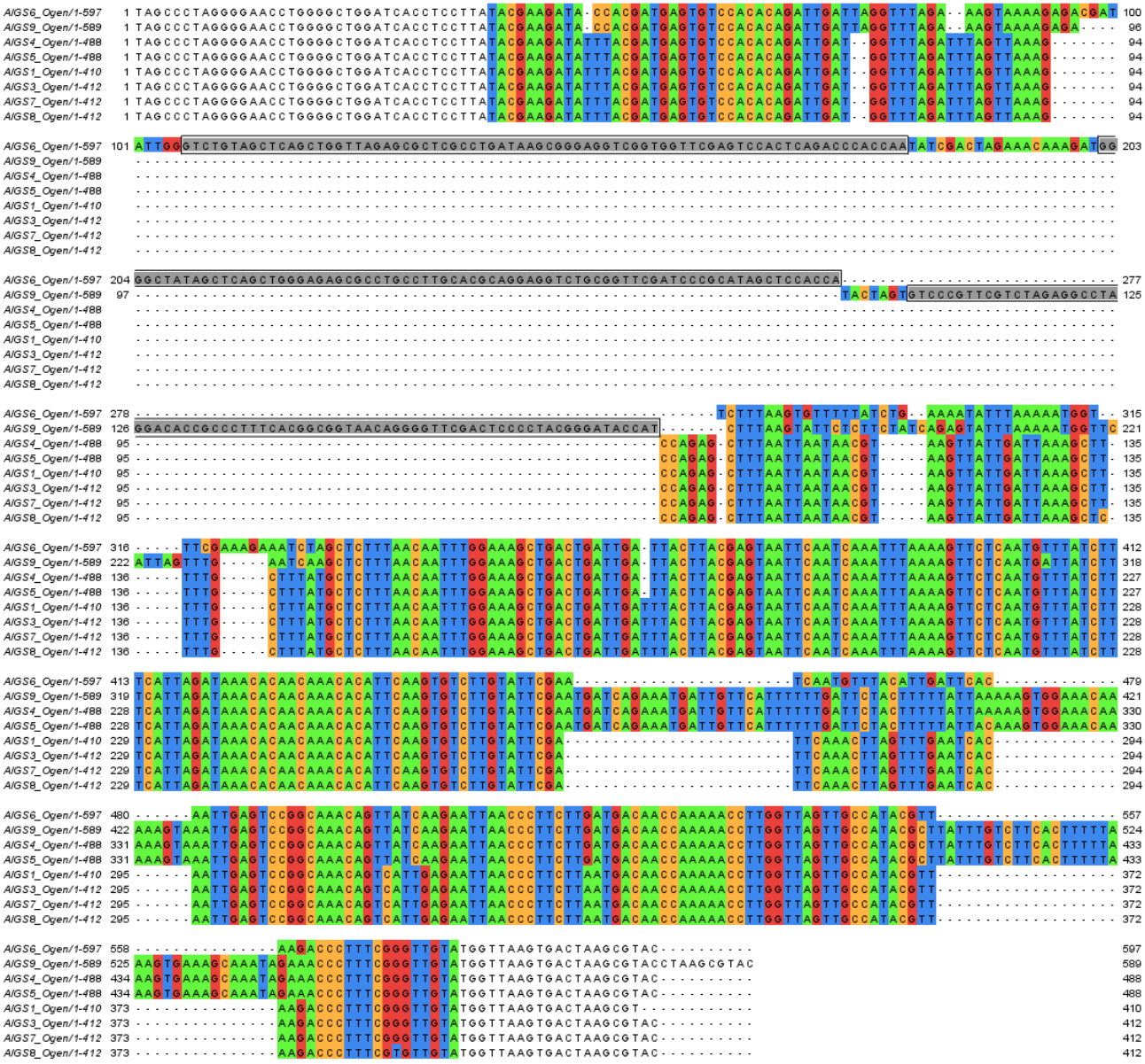


Figure 3.4: *Alignment of IGS sequences obtained from Vibrio Sample A, confirmed as Vibrio splendidus, illustrating the variation in IGS regions obtained from a single monoculture. Nucleotide sequence of 16S and 23S gene coding regions remain blank while IGS regions are coloured by nucleotide. tRNA genes are highlighted with grey boxes.*

3.5 Discussion

Results obtained from this research inform not only about the potential bacterial diseases that might result in fish following exposure to these jellyfish, information of high relevance to the aquaculture industry, but also the functionality of the adherent microbial community of jellyfish. The properties of each identified species of bacteria is discussed in this section.

3.5.1 Experimental technique

Various avenues of sequencing were explored in this study to identify to species level the genera of interest. This achieved species level resolution for the majority of isolates in a cost-effective manner without the need for whole genome sequencing. There are, however, a number of alternative methods that might have been employed.

3.5.1.1 Sample collection

Initial fieldwork by AK was conducted with the broad aims of collection of jellyfish medusae of *Phialella quadrata* and *Pelagia noctiluca,* due to their previous association with the salmon pathogen *T. maritimum,* which AK attempted to culture from jellyfish samples. Whilst success in this particular goal was not achieved, collection of *Cyanea capillata*, *Neoturris pileata* and *Obelia geniculata* along with water samples provided the opportunity to investigate the adherent microbial community of hereto unexplored cnidarian species. Two of these jellyfish (*C. capillata and O. geniculata*) have been previously associated directly with impaired health and survival of salmonids (Kintner and Brierley, 2018; M. D. Powell et al., 2018), and to our knowledge this work represents the first investigation of the microbial communities of *N. pileata* and *O. geniculata*.

The protocol of rinsing using sterile sea water to remove loosely associated environmental microorganisms mirrors that of other similar publications (Hao et al., 2015). The differentiation between what is a ‘true’ component of the adherent microbial

community and what is an environmental bacterial species that happens to be present on the jellyfish surface is a difficult distinction to make. It is possible many bacteria are both present in resident microbiomes and the surrounding environment. Use of the sterile wash step does, however, ensure that jellyfish-derived isolates were truly adherent to the tissue surface and not tank contaminants or coincidentally associated environmental populations. Although number of isolates for investigation was limited by exclusion of environmental isolates identical to host-derived, and likely meant real residents of the microbiome were lost to analysis, it did strengthen the assertion that bacterial isolates investigated here were true residents of the adherent populations of these jellyfish.

3.5.1.2 Culture

Culture was utilized in this work by AK as a way of separating different bacterial populations from an initial polyculture. Use of a culture step however crucially limited the conclusions that can be drawn regarding overall bacterial diversity of samples. Culture, particularly targeted culture, has long been understood as a step that reduces bacterial yield relative to high through-put molecular techniques (Hiergeist et al., 2015; Mcdonald et al., 2017; Staley, 1985) (Zimbro et al., 2009), particularly in marine bacteria where a large number are considered viable but not culturable (Belkin et al., 2005; Joint et al., 2010) . These bacteria might have been deteected direct investigation using techniques such as next generation sequencing or FISH.. Use of next generation sequencing would not have excluded cryptic populations of bacteria in the same way culturing does, and so might have been a more appropriate method of investigation, allowing a wider-ranging and thorough assessment of bacterial diversity (Salipante et al., 2013).

Although the culture step was tailored by AK to facilitate grown of Flavobacteriaceae for identification of *T. maritimum*, this species was not observed (Pazos et al., 1996). Failure to isolate *T. maritimum* may have been due to a number of factors. In culture, prolific species out-compete slower growing isolates, or those representing an initial lower percentage of the community composition (Austin et al., 2012; Belkin et al., 2005). An

additional consideration is the removal of bacteria-to-bacteria interactions within a biofilm by plate culture (Joint et al., 2010). Although absence from plate culture therefore does not determine true absence from the adherent communities of these jellyfish, investigation of the isolates obtained did still yield interesting results regarding the adherent microbiota of jellyfish.

3.5.1.3 Sanger sequencing

Use of culture to obtain microbial monocultures from the initial polycultures of bacteria facilitated separation and identification of individual bacterial isolates. Sanger sequencing of the 16S gene was determined to be the most cost effective and efficient method of taxonomically classifying isolates to allow identification of those of interest for further species-level sequencing. Primers for further species-level sequencing were selected for their utility in distinguishing all members of a genus to species level. Species-specific primers targeted at identifying major pathogens of aquaculture, such as *Aeromonas salmonicida*, *Vibrio anguillarum* and *Pseudomonas anguilliseptica,* are useful in disease diagnosis, and would have provided unambiguous classification of these species if present. Species-specific sequencing would though have failed to classify the majority of isolates obtained, including secondary pathogen *Pseudomonas fluorescens*, disease causing agent in cleaner fish species *Vibrio splendidus,* and isolates of potential interest in the jellyfish lifecycle.

3.5.1.4 Alternative methodologies

Next generation sequencing represents an alternative methodology that might have been utilised in this research, avoiding a culture step. However, despite the assurances of some publications to the contrary, the 300-500bp fragment of the 16S gene obtained by this method is generally insufficient for species-level taxonomic classification of bacteria, and often fails to obtain genus-level resolution for a proportion of results too (Tawfik et al., 2018). Had next generation sequencing for genus-level identification been employed in place of culture and Sanger sequencing of the 16S gene, samples would also have remained as mixed microbiota, not individual isolates classified to species-level.

Ultimately, next generation sequencing answers a different question than the one employed in this chapter, and would have provided different, albeit interesting information regarding the diversity of cnidarian microbiota.

Use of culture is suited particularly to antimicrobial testing (Austin et al., 2012) and phenotypic characterization towards full description of novel bacteria (Joint et al., 2010). Whilst the use of a culture step in this protocol has undoubtedly excluded bacterial genera and species from the final results, it achieved the overall aim of isolation of a number of bacteria for further investigation. The class that appears to dominate adherent microbial communities of Cnidaria, Gammaproteobacteria, contains species known to be associated with rich organic substrates (Tinta et al., 2012), and as such, is actually well suited to culture. The adherent microbes of jellyfish might therefore be broadly suited to culture, however employing a culture step for obtaining monocultures from mixed bacterial populations cannot be recommended, as culture precludes any conclusions regarding the absence of certain bacterial species.

3.5.2 Genus level identification

For the samples matching bacterial species within the Genbank database all belonging to the same genus, genus level classifications could be made. For the remaining samples, where Genbank matches were from multiple genera or even multiple orders, only higher-level taxonomic classifications could be assigned (**Figure 3.1**).

Initial 16S sequencing was dominated by the phylum Proteobacteria, with low numbers of Firmicutes and Actinobacteria. No bacteria of the Fusobacteria, Spirochetes, Bacteroidetes or Chlamydiae phyla could be cultured. No bacteria of class Mollicutes, a common phyla observed within the microbiome of *Aurelia aurita* jellyfish (Weiland-Brauer et al., 2015), were cultured either. All of the identified Proteobacterial isolates fall within the Class Gammaproteobacteria, dominant bacterial class on the surfaces of multicellular eukaryotic organisms (Hao et al., 2015; Schmitt et al., 2012; Sfanos et al., 2005). In jellyfish of the Class Scyphozoa, to which *Cyanea capillata* belongs, Alpha and Gammaproteobacteria appear to dominate bacterial populations in all jellyfish life-stages

(Weiland-Brauer et al., 2015). A study investigating the impact of jellyfish introduction on bacterial community composition change found that the majority of Scyphozoan-associated bacteria populations were culturable, with communities composed of mainly Gammaproteobacteria alongside Flavobacteria (Tinta et al., 2012). Whilst the identification of mainly Gammaproteobacteria from all isolates is undoubtedly biased by the culture technique used, which was targeted for marine fish pathogens, results of genus level comparisons are largely in agreement with existing literature on cnidarian microbiota (Abouna et al., 2015; Cleary et al., 2016; Kramar et al., 2018). Interestingly, different species were obtained from different body compartments of *C. capillata* in this study, including *Vibrio splendidus* from the gastric microbiota, but not the tentacle surface populations. Due to the limited number of monocultures obtained from each jellyfish individual, samples were grouped by species, with no individual and compartment specific comparisons, however this representing an area of potential future research.

Of the identified genera, a greater number were identified from the tissue of *C. capillata*. This may be as a function of a greater complexity of the microbiota of this jellyfish, however when considering these results, it is important to remember the limited sample size of jellyfish individuals, and that a culture step precludes conclusions regarding the diversity of microbial populations. Of the twelve genera identified using 16S sequencing, three were investigated further for species level resolution. These genera were selected on the basis that they contain bacterial species associated with disease in salmonid aquaculture in the Atlantic/North Sea waters of Scotland where samples were obtained.

The limitation of 16S is in taxonomic classification below the level of genus. Even full length sequences can fail to distinguish between species within a genus. Sequencings of 16S alone, even when the entire 1500bp gene is obtained, is also often insufficient for species level resolution between closely related *Pseudomonas* species, as well as *Vibrio* isolates (Alsina et al., 1996; Romanenko et al., 2008; Yamamoto et al., 2000). Species level differentiation could therefore only be achieved with further sequencing, for example of faster evolving, more divergent protein-encoding genes (Papke et al., 2003; Pernthaler and Pernthaler, 2005).

3.5.3 Species level identification

This study was conducted in a manner that provided identification of pathogenic microbes of interest alongside isolates not previously associated with fish disease within investigated genera. Through sequencing conserved genomic regions known to be sufficiently variable for species classification within the genus of interest, the majority of isolates were assigned taxa, not only those of pathogenic bacteria.

Despite the existing issues regarding rigid definitions of bacterial species, comparison against the Genbank database using the parameters of 100% coverage and at least 97% identity is the generally accepted method of genomic classification of bacteria. Classification to species level based on these parameters was therefore possible for the majority of isolates investigated. Whilst the potential implications of the presence of disease-causing isolates on the surface of jellyfish will be discussed, a number of caveats must be considered. First, presence or exposure to a pathogenic isolate does not necessarily lead to the onset of clinical disease – this means that exposure of fish to jellyfish carrying harmful bacteria will not automatically trigger an outbreak of bacterial disease. Disease initiation is a complex process influenced by more than just exposure to harmful bacteria. Secondly, previous association of certain microbes with clinical disease in salmonids does not assure the pathogenicity of all isolates. Bacteria have a very fluid genomic repertoire, so pathogenicity can vary greatly between strains of a species (Deng et al., 2003; Labreuche et al., 2006; Pemberton et al., 1997). Pathogenicity of the bacteria identified in this study cannot be ascertained without exposure of fish to these bacteria. The standard model of infectivity trials in fish involves either immersion in, or injection of bacteria, which are not ideal replications of the routes of infection for bacteria from Cnidaria. Future work might try to model the cnidarian vehicle for microbial delivery.

3.5.3.1 Aeromonas

16S sequencing identified two isolates as *Aeromonas*. Further characterisation was performed using primers targeted at the protein-encoding DNA gyrase subunit B gene, as part of the enzyme DNA gyrase (Filutowicz and Jonczyk, 1983). This gene has a

substitution rate greatly in excess of that of the 16S gene, giving it higher variability and therefore an increased likelihood of nucleotide differences for distinguishing between species (Yamamoto and Harayama, 1996). It has been used previously to successfully classify *Aeromonas* isolates to species level (Sen, 2005; Yáñez et al., 2003) and was therefore selected as the sequence for classification of our samples.

Aeromonas salmonicida

A. salmonicida is a known bacterial pathogen of fish, here isolated from the adherent microbial community of *C. capillata* gut. Whilst the scope of this investigation did not extend to challenge trials and infection of fish to ascertain the pathogenicity of isolates, identification of *A. salmonicida* from *C. capillata* is an important finding for a number of reasons.

A. salmonicida is the causative agent of furunculosis, arguably the bacterial infection with the most significant commercial impact on freshwater and marine salmonid aquaculture, impacting the salmonid industry in all countries except Australia and New Zealand. Described subspecies include *salmonicida* (causative agent of 'typical' furunculosis), *achromogenes, masoucida, pectinolytica* and *smithia* (pathogens associated with 'atypical' furunculosis). Outbreaks of all typically occur in warmer temperatures (>10°C) and following fish stressors such as handling or smoltification. Presentation can vary, however the gross symptoms of both typical and atypical furunculosis include furuncles progressing to crater lesions, multifocal haemorrhaging and bloody discharge. Gill histology is characterised by the presence of rod-shaped, gram negative bacterial colonies alongside lamellar fusion, necrosis of the epithelium and eosinophilic inflammatory infiltration (Bruno et al., 2013; Jangoux, 1986; Wooster and Bowser, 1996).

Aeromonas molluscorum

A. molluscorum is a species of bacteria that was first isolated from bivalve molluscs (Miñana-Galbis et al., 2004), not previously associated with disease in fish (Cruz et al., 2013) Review of the available literature suggests that our data represents the first isolation

of this bacterium from the adherent bacterial communities of jellyfish.. Resistant strains of *A. molluscorum* previously misclassified as *Aeromonas veronii* are known to proliferate in the presence of the biocide tributyltin, utilised historically in anti-biofouling paints (Cruz et al., 2010, 2007). Given the persistence of this toxic chemical in the environment, particularly in marine sediment (Al-rashdi, 2011; Dafforn et al., 2011), degradation of this compound might prove a highly advantageous trait for aquatic organisms. Whilst medusae are free swimming, *A. molluscorum* was isolated from the tentacles of *C. capillata*, a jellyfish with a sessile polyp life stage. Sessile life stage settlement is known to be affected by bacterial populations, which can make the difference between success or failure (Neumann, 1979).

3.5.3.2 Pseudomonas

Sequencing of the 16S gene identified 14 potential *Pseudomonas* isolates from jellyfish tissues. Some samples matched with a high level of similarity to multiple genera, and although the majority of matches were *Pseudomonas*, these samples could only be classified to a higher taxonomic level. These samples were treated as suspected *Pseudomonas* samples and included in the additional sequencing required for species level resolution. Sequences of the *rpoD* gene, a sigma factor for promotion of the attachment and release of RNA polymerase at initiation sites, has been conducted successfully previously for species-level resolution of unknown *Pseudomonas* isolates (Glaeser and Kämpfer, 2015; Mulet et al., 2009a), and was therefore adopted for this study.

Table 3.2 Taxonomic summary and sequencing accession numbers

Sample Identifier	Origin	Genus level identification	Accession of 16S sequence	Species level classification	Accession of further sequencing
Ae1CcapG	*C. capillata, gut*	*Aeromonas*	MH205967	*A. molluscorum*	MH310838
Ae2CcapT	*C. capillata, tentacle*		MH205968	*A. salmonicida*	MH310839
A9Ogen	*O. geniculata*	*Vibrio*	MH205969	*V. splendidus*	MH310852
					MH310853
					MH310854
					MH310855
					MH310856
					MH310857
					MH310858
					MH310859
B5Ogen	*O. geniculata*		MH205970	*V. splendidus*	MH310860
					MH310861
C4Npil	*N. pileata*		MH205971	*V. alginolyticus*	MH310862
					MH310863
					MH310864
D7Npil	*N. pileata*		MH205972	*V. splendidus*	MH310865
					MH310866
					MH310867
					MH310868
					MH310869
					MH310870
					MH310871
E2Npil	*N. pileata*		MH205973	Novel strain	MH310872
					MH310873
F3CcapG	*C. capillata, gut*		MH205974	Novel strain	MH310874
					MH310875
					MH310876
G3Ogen	*O. geniculata*		MH205975	*V. crassostreae*	MH310877
					MH310878
					MH310879
H3CcapG	*C. capillata,* gut		MH205976	*V. splendidus*	MH310880
					MH310881
					MH310882
P3Ogen	*O. geniculata*	*Pseudomonas*	MH205979	*P. fulva*	MH310842
P7CcapG	*C. capillata,* gut		MH205983	*P. fluorescens*	MH310846
P8CcapT	*C. capillata,* tentacle		MH205984	*P. fulva*	MH310847
P9Npil	*N. pileata*		MH205985	*P. fulva*	MH310848
P1CcapT	*C. capillata,* tentacle		MH205977	*Pseudomonas* sp.	MH310840
P2CcapT	*C. capillata,* tentacle		MH205978		MH310841
P4Ogen	*O. geniculate*		MH205980		MH310843
P5CcapT	*C. capillata,* tentacle		MH205981		MH310844
P6CcapG	*C. capillata,* gut		MH205982		MH310845
P10Ogen	*O. geniculate*		MH205986		MH310849
P11Ogen	*O. geniculate*		MH205987		MH310850
P12Ogen	*O. geniculata*		MH205988		MH310851

Table 3.2*: Accession information and taxonomic classification of isolates classified to species level in this chapter. The majority of isolates were classified using BlastN against the Genbank database, however a small number of Pseudomonas could not be definitively classified this way, and two Vibrio isolates appear to be suitably different from existing submissions to as represent novel strains (however this may be due also to lack of suitable comparison material online).*

Comparison of sequences generated using the *rpoD* primers, using BLASTN against the Genbank database, initially identified 43% of samples to the species level. These were all classified as *Pseudomonas fulva* and *Pseudomonas fluoresces,* with the exception of two samples that were determined to not be *Pseudomonas*: *Pseudoalteromonas aliena* and *Acinetobacter guillouiae*. The *rpoD* primers utilized for this research were not genus-specific to *Pseudomonas* (Yamamoto et al., 2000, 1999), so this was not unexpected. Phylogenetic analysis suggested close relationships for a further four samples with *Pseudomonas alkylphenolia* and *Pseudomonas guineae*. A failure to identify the remaining four isolates to species level was almost certainly influenced by the lack of available curated type strain *Pseudomonas rpoD* sequences. Whilst a large amount of high-quality information is available for the 16S gene, there is significantly less verified sequencing data for genes such as *rpoD*.

The genus *Pseudomonas* contains many described species, the exact number of which varies depending on the information source: The 'Complete List of Prokaryotic names with Standing in Nomenclature' (http://www.bacterio.net/pseudomonas.html) lists 242 species of *Pseudomonas* with 18 subspecies. 'Strain Info' has 233 entries including subspecies (http://www.straininfo.net/taxa/1186), and the *Pseudomonas*-specific curated PseudoMLSA database contains 146 entries (http://microbiologia.uib.es/bioinformatica/Pseudomonas_CompleteList.php?textfield=) . It is therefore almost impossible to obtain a truly comprehensive list of *Pseudomonas* species and subspecies for the purposes of constructing a phylogenetic tree. The *rpoD* phylogenetic tree utilised in this study (**Figure 3.3**) was constructed using *Pseudomonas* type strain *rpoD* sequences available at the time. Sequences were obtained from the PseudoMLSA database in an effort to ensure quality and curation. Every effort was also made to include all clinically relevant isolates, particularly those responsible for disease within the 40-70-degree latitudes of the northern hemisphere and 40-50-degree latitudes of the southern hemisphere, which are associated with salmonid production. This included *Pseudomonas fluorescens* and *Pseudomonas plecoglossica* (Austin et al., 2012; Sakai et al., 1989), *Pseudomonas alcaligenes* (Starliper, 2013), *Pseudomonas anguilliseptica* (Austin et al., 2012), *Pseudomonas putida* and *Pseudomonas beatica* (López et al., 2012). Failure to include every species and type strain does however

introduce a level of ambiguity for definitive categorisation of microbes. Tree construction therefore provides an idea of taxonomic association, but not definitive species classification.

Construction of a phylogenetic tree using uncurated *Pseudomonas rpoD* sequences looks quite different to the 'curated' tree generated for species-level resolution. The differences could be attributable to incorrect taxonomic classifications, variable sequence quality and uncurated nature of many sequences. It was decided to use the tree of curated sequences, because despite the lower number of species included, the information was considered more reliable.

Pseudomonas fluorescens

Pseudomonas fluorescens, here isolated from *C. capillata*, is a common environmentally isolated marine bacterium (Scales et al., 2014) frequently identified in organic biofilms, within sediment or suspended in the water column. Although rarely a primary pathogen, it has been associated with secondary infection following previous trauma, stress or pathogenic insult (Austin et al., 2012), notably in juvenile Atlantic salmon (Hjeltnes et al., 2018). It can also produce the compound phenazine, which has a role in surface attachment in biofilms (Maddula et al., 2006), and other extracellular products with antimicrobial and antifungal properties, such as pyoluteorin and 2,4-diacetyl-phloroglucinol. These products might be useful to cnidarians in colonisation of the marine environment, and bioremediation (Holmström and Kjelleberg, 1999; Sarniguet et al., 1995; Schnider et al., 1995). The ability of bacteria to act as bio mediators greatly assists in their success within a biofilm or microbiome, preventing growth of competing bacteria (Mavrodi et al., 2006). *P. fluorescens* might therefore represent an important modulatory organism within the jellyfish microbiome, as well as potential opportunistic pathogen of fish.

Pseudomonas fulva

P. fulva is considered a part of the *P. putida* group in the *Pseudomonas fluorescens* lineage, closely related to *P. parafulva* and *P. cremoricolorata* (Mulet et al., 2012). There is little literature regarding the activity or ecological role of *Pseudomonas fulva*. With no documented cases of disease in any species of fish, this isolate has been considered as non-pathogenic to salmonids and concurrently cultured species. It is, however, worth noting the involvement of *P. fulva* in human septicaemia infections (Almuzara et al., 2010). It also appears to have a protective influence against fungal infections when present in the microbiota of plants (Strzelczyk and Li, 2000).

3.5.3.3 Vibrio

Within the literature there exist a number of methodologies for determination of *Vibrio* isolates to species level and sequencing of the IGS region was selected based on an extensive literature review. *Vibrio* possess multiple IGS regions throughout their chromosome, and as a result PCR of this region produces a mixed product of different fragments that require to be separated via plasmid sub-cloning prior to sequencing. This was advantageous for species level classification in this study, as multiple sequences provide a greater level of resolution for taxonomic classification. Some obtained sequences proved to be diagnostic to the species level and others did not, highlighting the need for sequencing multiple fragments. Overall results suggest a number of IGS sequences are shared across species of Vibrio, whereas some are unique to individual species.

Vibrio splendidus and Vibrio crassostreae

V. splendidus and *V. crassostreae* dominate the species of *Vibrio* identified in this study. These species of vibrio are closely related, with *V. crassostreae* recently differentiated (Faury et al., 2004). *V. splendidus* has become a bacterium of concern to the aquaculture industry due to its suspected association with mortalities in ballan wrasse and lumpfish, used as ‘cleaner fish’ in biological control of sea lice in Atlantic salmon production

(Bornø et al., 2016; Gulla et al., 2015; A. Powell et al., 2018). Bacterial disease appears to be associated with the high losses in these species (Nilsen et al., 2014). Previous to this recently discovered association with cleaner fish death, infection with *V. splendidus* was only observed in crustaceans and molluscs (Chatterjee and Haldar, 2012; Romalde and Barja, 2010), in which it also causes infectious disease (Dubert et al., 2017).

Vibrio alginolyticus

Vibrio alginolyticus is a bacterium associated with fish pathology, causing species-nonspecific Vibriosis (Caremen Balebona et al., 1998; Carmen Balebona et al., 1998; Rajan et al., 2001). This disease is characterized by septicaemia, haemorrhage and ulceration of epithelia (Caremen Balebona et al., 1998). Vibriosis remains a serious problem for global salmon aquaculture, particularly in the marine environment (Meyers et al., 2008). Whilst *V. anguillarum* has historically been the main species of concern, particularly for salmonids, *V. alginolyticus* has been shown to be cytotoxic to Chinook salmon (*Oncorhynchus tshawytscha*) (Carmen Balebona et al., 1998; Meyers et al., 2008).

Unknown Vibrio

A number of papers report isolation of bacteria of unresolved taxonomic classification, due to insufficient resolution from the sequence data. There are reported to be a great number of as yet unclassified bacterial isolates, particularly in the marine environment (Ivanova et al., 2003; Schuett and Doepke, 2010; Venkateswaran et al., 1998). There is certainly less available sequencing data for the IGS region than more commonly targeted genes, such as 16S. Unclassified isolates observed in this study may well be novel species of *Vibrio*, however they may also represent previously described species of *Vibrio* for which IGS sequences are not available within Genbank. Regardless, confirmation by phenotypic characterization and ideally whole genome sequencing is required to formally describe a new species (Schumann et al., 2009).

3.5.4 Potential roles in the jellyfish lifecycle and microbiome

Previous research has highlighted mutualisms between adherent bacteria and their cnidarian hosts (Abouna et al., 2015; Cavanaugh, 1994; Schuett et al., 2007; Zarubin et al., 2012). One of the most well documented marine symbioses is between coral and cyanobacteria, which aid in nitrogen fixation (Charpy et al., 2012), but recent research highlights potential marine symbionts of cnidarians for metamorphosis, settlement and even toxin formation (Neumann, 1979; Schuett and Doepke, 2010; Weiland-Brauer et al., 2015). Bacterial species *A. molluscorum* and *P. fluorescens* identified in this study represent microbes as part of the cnidarian microbiome, with potentially important functions in the survival and success of their host. Settlement and growth of polyps can be inhibited by anti-biofouling paint or environmental biofilms (Fitridge et al., 2012), so the possession of antimicrobial peptides and the ability to proliferate in anti-biofouling environments. Jellyfish such as *Cyanea capillata* and *Obelia geniculata* have sessile life stages that grow in competition with other seabed organisms, so an advantage conferred by bacterial mutualisms could be important. *A. molluscorum* might facilitate proliferation of polyps in anti-biofouling environments such as underwater architecture of fish farms (Cruz et al., 2015, 2013). Antimicrobial peptides and adherence factors such as are produced by *P. fluorescens* might prevent overgrowth of other bacterial potentially harmful to the host (Martı et al., 2018; Mavrodi et al., 2006; Noga et al., 2011; Ostaff et al., 2013) as well as assist in cnidarian settlement (Costerton et al., 1987; Liu et al., 2016; Solano et al., 2014). *P. fluorescens* was observed across the cnidarians sampled. Its shared presence might then imply a shared functional requirement by hosts.

Venom composition varies between jellyfish species (Burke, 2002; Carli et al., 1996) as well as between individuals of the same species with geographical distribution, life stage (Helmholz et al., 2010, 2007; Wiebring et al., 2010) and even body compartment (Helmholz et al., 2007; Kramar et al., 2019). If bacteria are involved in venom production, this might go some way to explaining these differences. Mutualistic relationships between aquatic animals and bacteria for toxin production is after all an established phenomenon. Bryostatin toxin is produced by the bacterial symbiont *Candidatus endobugula sertula* (Flórez et al., 2015; Haygood et al., 1999), Tambjamine-producing *Pseudoalteromonas*

bacteria are utilized by nudibranchs, bryozoan and tunicates for chemical defence (Flórez et al., 2015) and endosymbiont production of tetrodotoxin (Noguchi et al., 1987) occurs in a taxonomically varied group of marine organisms (Lee et al., 2000; Maruyama et al., 1984; Wu et al., 2005; Yasumoto et al., 1986). Various bacterial genera implicated in tetrodotoxin production, including *Pseudomonas* (Yotsu et al., 1987), *Bacillus* (Wu et al., 2005), and *Vibrio* species (Yu et al., 2004). Of particular note in its mutualistic association with puffer fish is *Vibrio alginolyticus* (Noguchi et al., 1987; Thuesen and Kogure, 1989), a bacterial isolate identified in this study. Endobiotic *Moritella viscosa,* identified within tentacle tissue from *Cyanea lamarckii,* (Schuett and Doepke, 2010) produces cytotoxic extracellular microbial products as part of 'winter ulcer disease' in salmonids (Bjornsdottir et al., 2011). *Aeromonas* identified here also synthesises harmful extracellular products, including nucleases, proteases, cytolytic toxins, lipases, sulphatases, lecithinase and amylase that degrades tissue structures (Austin et al., 2012; Pemberton et al., 1997). The enzymatic action of *C. capillata* venom includes cytotoxic and haemolytic activity (Brinkman and Burnell, 2009; Jouiaei et al., 2015b; Šuput, 2011), characteristics shared with furunculosis and vibriosis infection (Helmholz et al., 2007). Infection with *Aeromonas* and *Vibrio* causes erosive tissue diseases not unlike that of *T. maritimum*, the proteolytic fish pathogen already linked to vector transmission by jellyfish (Delannoy et al., 2011; H. W. Ferguson et al., 2010).

More research is needed into both the core microbial communities and venom chemical composition of individual cnidarians before any definitive conclusions regarding the association of adherent bacteria and toxin production can be drawn. It is, however, interesting that the potentially pathogenic bacteria now isolated from medusoid jellyfish (*T. maritimum*, *A. salmonicida* and *Vibrio* species) are all associated with tissue necrotizing, ulcerative pathologies, which can be considered as having certain mechanistic similarities to jellyfish prey digestion.

3.5.5 Vector Transmission

There is little information available regarding bacterial infections in cnidarians themselves. Reports of necrotic lesions in jellyfish hint at bacterial diseases in medusae, with descriptions of bell damage and subsequent bacterial digestion of tissue a common

theme (Ladouceur et al., 2012; Steers et al., 2003; Williams et al., 2011), however fail to identify infectious microbial disease. The suggestion is that bacterial infections occur infrequently in medusozoan jellyfish, perhaps due to their body composition, which is high in water and low in cellular components, with overall low nutritional value (Davenport, 1998; Doyle et al., 2007b). Bacteria present on the tissue of the animals are therefore unlikely to be present as part of an ongoing disease of the jellyfish itself, and more likely a component of the normal adherent microbiota. Certainly, jellyfish recruit their microbial populations from somewhere, likely each-other and the surrounding marine environment. These bacteria are not then confined to the jellyfish tissue though, and transfer must occur in both directions, from and to tissue.

Medusozoan jellyfish can travel large distances (Doyle et al., 2007a; Lynam et al., 2004), and small hydrozoan species often propagate locally in coastal environments where marine aquaculture occurs, even growing on the underwater structures of the farm itself (Guenther et al., 2010; Hodson et al., 1997). Passage of these jellyfish into the caged environment brings with them their adherent microbial communities, and with them, potential pathogens of salmon aquaculture. Potentially pathogenic microbes isolated from the cnidarian microbiome in this research, including *A. salmonicida* and Vibrio species, might therefore by transferred from jellyfish to farmed salmonids. This transfer and the lack of routine monitoring of jellyfish populations might partially explain the phenomenon of marine outbreaks of furunculosis, a disease often associated with vector transmission. Wild fish and sea lice are considered within biosecurity planning for aquaculture production sites; however, jellyfish rarely are. Given the added factor of predisposition to bacterial infection by sting-induced trauma, jellyfish as vectors of bacterial disease must be considered a threat to salmonid production.

Whether the bacteria identified in this study are core elements of the microbiomes of *C. capillata, O. geniculata* and *N. pileata* remains to be evidenced, however, it can be concluded that these cnidarians are viable hosts for the bacteria identified from each.

3.6 Conclusions

This is the first study, to our knowledge, investigating the adherent bacterial communities of *O. geniculata* and *N. pileata*. The results inform not only regarding the presence of potentially pathogenic microbes within the adherent microbial communities of these jellyfish, but also in the non-pathogenic taxa as part of tissue populations. While species associated with erosive tissue pathologies might play a role in venom activity, these non-pathogenic bacteria might too play a role in the lifecycle of their cnidarian hosts. Further work might focus on the exploration of the microbiota of these jellyfish using metagenomic sequencing techniques to further understand the community composition and functional roles of the adherent microbiota of these species. With reported increasing populations of jellyfish globally, a greater understanding on their microbial biology might aid in local control and mitigation strategies. Important too, with the historically careless use of antibiotics and growing resistance of infectious disease, is the current focus of scientific research in discovery of antimicrobial compounds. The marine environment, and cnidarians in particular, appear an exciting avenue of exploration in this field (Mariottini and Grice, 2016; Ovchinnikova et al., 2006; Tortorella et al., 2018), with novel marine microbes and their products considered a promising source of compounds with pharmaceutical applications (Wiese and Imhoff, 2019).

C. capillata, commonly known as 'lions mane', is a large jellyfish with global distribution commonly seen in UK waters in June-September (Doyle et al., 2007a) and is well known for its ability to painfully sting humans (Burke, 2002). *N. pileata* and *O. geniculata* are smaller jellyfish species that cannot be observed in the marine environment without magnification. Work resulting from the PhD of AK links *O. geniculata* in particular to gill disease in Atlantic salmon (Kintner and Brierley, 2018). Whether they are capable of traumatizing delicate gill tissue directly remains to be demonstrated, however their association with increased fish mortalities by Kintner et al points to a negative impact of exposure (Kintner and Brierley, 2018). Dysbiosis or introduction of pathogenic bacteria to the gill microbiome by exposure to jellyfish might go some way to explaining the impact of microscopic Cnidaria such as *Obelia* (Kintner and Brierley, 2018).

Wild fish and parasites such as sea lice are understood to be vectors of bacterial disease, however the risk of jellyfish in the transmission of pathogens is rarely considered by aquaculture professionals (Barker et al., 2009; Belkin et al., 2005; Cusack and Cone, 1986; Nylund et al., 1991), despite the previous association of jellyfish exposure in Tenacibaculosis initiation. *T. maritimum* is also not alone in its capacity to elicit epithelial infections in fish species, and this work identified a number of other bacteria associated with erosive tissue infections in salmonids. These potential aquaculture pathogens were hosted within cnidarian microbial populations, and without exclusion of jellyfish from the net environment, producers risk exposing fish. Although conclusions regarding the role of the identified bacteria as core microbiota or as part of a variable, more transient community cannot be drawn, this work demonstrates that these jellyfish can certainly host harmful bacteria beyond previously described *T. maritimum*. The pathogenic potential of isolates obtained in this study was not fully determined, for reasons explained above. A next step from this research could therefore be for an exposure trial of fish to *A. salmonicida* or *V. alginolyticus* obtained from jellyfish microbial populations. It will, however, be difficult to replicate the circumstances of exposure without inclusion of intact jellyfish specimens in such as trial. Nevertheless, this study demonstrates the ability of jellyfish to host bacteria associated with pathogenic disease within their adherent microbial populations. They could, therefore, be acting as vectors of disease transmission for infectious bacteria to salmonids and other aquaculture-reared marine species. The established traumatic consequences of exposure of fish to jellyfish such as *C. capillata* elicits stress and tissue lesions, factors that may precede and predispose fish to bacterial infection. The analogy therefore proposed is that of a ‘dirty needle’, wherein cnidarian trauma coincides with exposure of fish to the microbiota of the stinging apparatus.

Adherent bacteria on the tissue of jellyfish will come into contact with farmed fish when jellyfish are present in the net environment. By demonstrating the presence of potentially pathogenic bacterial isolates, this research enhances our understanding of the role jellyfish may play in vector transmission and initiation of bacterial disease. Without exclusion of jellyfish from the net environment this cannot be mitigated, and without accurate routine sampling the true impact of ongoing exposure cannot be estimated.

Large distinctive jellyfish such as *C. capillata* are easily identified, but rarely reported. Aquaculture facility records rarely include information regarding the presence of these jellyfish, and in what weather conditions. Hydromedusa are known to be common cnidarian zooplankton in coastal Atlantic waters (Baxter et al., 2012a), however very little routine sampling and identification is being performed. Without an understanding of what species are present, and in what numbers, the true impact of these organisms cannot be elucidated. Future research must inform on cnidarian species and the density at which a negative impact on fish health is observed. The findings of this study support the hypothesis of jellyfish as vectors of potential pathogens to salmonid aquaculture, and with improved sampling for these jellyfish their true involvement in unexplained outbreaks of bacterial disease might be better understood.

Chapter 4

4. Transcriptomic response of rainbow trout gills to toxin producing phytoplankton, *Prymnesium parvum*.

4.1 Abstract

Whilst phytoplankton are not directly infective organisms, harmful algal blooms (HABs) have a serious impact globally on both aquaculture production and wild fisheries (Díaz et al., 2019; Hinder et al., 2011; Rensel et al., 2010), with many major blooms and fish kills reported annually. The known pathological consequences of exposure to these organisms include systemic physiological impacts from altered water oxygen concentration, as well as toxin production and direct mechanical damage to gill tissue (Trainer et al., 2012; Wells et al., 2015). The consequences of exposure to these non-infective organisms in fish, particularly of sub-lethal sequelae, are currently still poorly understood. This work represents the first investigation into the mechanisms of tissue transcriptome response to toxin producing phytoplankton in salmonid fish. There does, however, exist a great deal of work on the immunological and toxicological response of salmonids, including common aquaculture species *Oncorhynchus mykiss* (rainbow trout), to other harmful agents. The majority of existing work in gills though is focused on the impact of infectious agents, with diseases such as AGD, Salmon Pox Virus or bacterial infection. The field is notably data deficient in research on agents of gill trauma other than infective pathogens, such as from phytoplankton or jellyfish insult, however perhaps parallels do exist.

This study investigated the transcriptomic response of gill tissue exposed to a toxin-producing phytoplankton, *Prymnesium parvum.* This involved exposing rainbow trout to two concentrations of toxin-producing *P. parvum* cells, designed to induce alteration of gill tissue gene expression. *P. parvum* is a phytoplankton of concern for rainbow trout production especially in Denmark and the USA, as it produces a mixture of organic toxins known to cause fish mortality. The gill transcriptome response to *P. parvum* in this study exposure showed over 1000 genes were altered, and subsequent gene set enrichment identified many pathways and processes impacted by this exposure.

Responses of note included activation of oxygen deprivation pathways, immune function and inflammation, cell death and antioxidant response. Dose effect differences were observed both clinically and in transcriptional response. Individuals within the higher concentration treatment group also demonstrated a divergence of response, both clinically and in gene expression, which was explored in results. Overall, results provide novel insight into the gill response to toxin exposure and mechanisms of *P. parvum* toxicology. Outputs from this research may provide tools and knowledge for managing impacts of HABs in farmed fish.

4.2 Introduction

Rainbow trout (*Oncorhynchus mykiss*) show both anadromous and non-anadromous life history types and are farmed in both fresh, brackish and marine environments (Kultz, 2015). Of increasing concern for production of this commercially important species is the gill damage and large fish kills that have been documented from exposure to toxic phytoplankton (Gatz, 2018).

4.2.1 *Prymnesium parvum* as a challenge to finfish production

Although phytoplankton do not directly attack fish for infective reasons, they elicit significant impairment of fish health and survival (Gatz, 2018). The majority of dinoflagellates photosynthesise and can significantly raise oxygen levels during daylight, however once a bloom dies they oxygen levels can drastically reduce through massive overgrowths in aerobic bacteria as part of the decomposition of phytoplankton. The challenge to farmed fish is particularly pronounced because of their inability to move away from a harmful environment in algal bloom situations, however these phytoplankton can kill wild stocks too. Phytoplankton can cause gill damage due to their cell structure, with species such as *Chaetoceros* or *Pseudo-nitzschia* causing irritation and physical damage of delicate gill lamellae (Dorantes-Aranda et al., 2011; Yang and Albright, 1992). This mechanical irritation of gills has been observed to lead to over-production of mucilage within the gills, leading to occlusion of the secondary lamellae of fish gills (Jones and Rhodes, 1994; Lumsden et al., 1994) and eventual suffocation.

Phytoplankton can also damage fish also through biotoxin production (Dorantes-Aranda et al., 2015; Rodger et al., 2010). The environmental factors involved are not yet fully understood , but toxin production by algae can result in mass fish kills (Doucette et al., 2005). These are known as Harmful Algal Blooms (HABS) and occur world-wide, with serious impact on entire ecosystems. Different phytoplankton produce different toxins, such as Diarrhoeic Shellfish Poison (DSP) from Okadaic acid in *Dinophysis* spp. (Reguera et al., 2014) and Neurotoxic Shellfish Poison (NSP) from Brevetoxins

producing *Karenia brevis* (Watkins et al., 2008). Many of these toxins are of public health concern due to their accumulation up trophic food webs, or within filter feeders such as bivalves (Visciano et al., 2016). Some HAB phytoplankton though also produce toxins with a direct harmful effect on fish.

P. parvum is one such toxin-producing phytoplankton. A dinoflagellate most commonly associated with brackish waters (Kaartvedt et al., 1991), the general consensus is that this phytoplankton produces a mixture of toxins that can have numerous negative consequences in exposed fish. First described associated with fish mortalities in Denmark in 1950 (Guo et al., 1996), this species is observed globally, impacting fish stocks in most countries where rainbow trout are farmed (Wagstaff et al., 2018), as well as other aquaculture species. It is possible too that, similar to zooplankton, a warming climate may cause an increased incidence of toxin-producing blooms, and exposure to these HABs may become an increasing problem for both aquaculture and wild fisheries.

4.2.1.1 Ecology of toxin production

P. parvum is known as a 'golden algae' due to its pigmentation. This species is highly successful in a variety of environments due to its euryhaline and eurythermal capabilities, as well as mixotrophic behaviour (Watson, 2001). This means it can photosynthesise, but is also able to phagocytose small organisms such as microalgae and bacteria to meet its energy requirements (Springer and Holley, 2013). Investigation of the toxic compounds produced by *P. parvum* is ongoing, with research suggesting that toxin production occurs as a by-product of imbalanced cell metabolism in nutrient deficient conditions (Dafni et al., 1972) or as part of an allelopathic function, assisting in the mixotrophic ability of *P. parvum* for capture of prey (Letters, 2012; Wagstaff et al., 2018; Yarev et al., 1961) and avoiding predation (Tillmann, 2003).

The toxins produced by *P. parvum* are diverse and have documented ichthyotoxic, cytotoxic, haemolytic, hepatotoxic, neurotoxic, antibacterial and allelopathic properties (Yarev et al., 1961). Compounds produced include proteolipids (Ulitzur and Shilo, 1964) such as haemolysin and lipopolysaccharide-like compounds, proteolipid

glycoglycerolipids (Kozakai et al., 1981), fatty acids and the polyether toxic compounds known as prymnesins (Igarashi et al., 1996; Manning and La Claire, 2010). The prymnesins are cyclic oxygen-containing toxins known as Prymnesin-1 and Prymnesin-2 , that can be further classified into subtypes (Binzer et al., 2019). These toxins are produced by *P. parvum* in minute quantities that make isolation and quantification very difficult. There is still some ambiguity even whether toxins are produced intracellularly and released only during stress, or whether toxins are present both intracellular and extracellular within the cells of *P. parvum*, with exposure to aquatic organisms during cell lysis (Freitag et al., 2011).

4.2.1.2 Impairment of fish health

P. parvum toxin exposure affects gill breathing aquatic species. The toxins appear to have no effect in organisms without gill tissue, and are thought to be inactivated by the acidity of the gastrointestinal tract in these animals on ingestion (Shilo and Aschner, 1953) . Toxins are thought to act principally on gill epithelial cells, either through direct lysis of cells (Tillmann, 2003; Yarev and Hestrin, 1961) or altered membrane potential and ion balance (Yarev et al., 1961). This then allows entry of additional toxic metabolites produced by this phytoplankton, as well as sensitisation of fish to other environmental toxins. Whilst many studies involved injection challenges or cell culture (Parnas, 1963; Ulitzur and Shilo, 1966) and do not inform on the action of *P. parvum* in an environmental exposure setting, do still provide valuable information regarding toxin activity, such as neurotoxic and cytotoxic effects (Dafni et al., 1966; Igarashi et al., 1996; Manning and La Claire, 2010). Despite a lack of consensus within previous publications regarding the specific components and action of *P. parvum* toxin, existing research overall suggests a mode of toxin activity in depolarisation via direct attachment of prymnesin toxins to gill tissue, leading to disruption of ion regulation, a loss of selective permeability and compromised gill integrity (Manning and La Claire, 2010). This primary activity of binding to gill tissue and altered permeability of gill cell surfaces appears to then allowing ingress and secondary activity of additional harmful toxin chemicals (Ulitzur and Shilo, 1966).

Observed cellular changes within the gill include swelling, followed by lysis (Dafni et al., 1966). The reported toxic effects of *P. parvum* in live fish are many, including haemolytic, anti-coagulative and ichytoxic consequences (Manning and La Claire, 2010) . The physiological effects of *P. parvum* exposure appear to include respiratory impairment of rainbow trout. Whilst initial functional impairment is reported to be reversible, continued exposure results eventually in death (Andersen et al., 2016; Svendsen et al., 2018). The histological changes following *P. parvum* exposure are poorly understood, however, it has been demonstrated that this phytoplankton impairs gill functionality, and is a suggested tissue irritant (Svendsen et al., 2018).

4.2.2 Transcriptomics of toxin response

Existing research has studied the clinical impact of *P. parvum* toxins on fish gills, but no study has yet investigated the transcriptional response of gill tissue. Little information is available regarding even general molecular mechanisms of disease in gill-breathing fish following exposure to toxin-producing phytoplankton. Most research that does exist is focused on phytoplankton such as those within the *Chatonella* and *Alexandrium* genera, associated with HABs, and the response of bivalves. *Chatonella* has been demonstrated to produce reactive oxygen species and kill fish directly rather than through bioaccumulation (Ishimatsa et al., 1997), with a suggested mechanism of gill damage similar to that of *P. parvum* (Tiffany et al., 2001) . Of the studies regarding impact of *P. parvum* exposure specifically in teleosts, none investigate the transcriptomics of response most (focused instead on physiological alterations and clinical presentation in fish) (Guo et al., 1996; Watson, 2001).

Although the transcriptome response has not yet been described for *P. parvum* exposure in fish, many of the pathways of response to other harmful stimuli appear shared in rainbow trout and might occur too in response to exposure to this harmful organism. Gill response pathways to stimuli such as stress or hypoxia are well described in rainbow trout (Iftikar et al., 2010; Soitamo et al., 2002; Uren Webster et al., 2018). Infectious agents elicit specific and non-specific immune responses (Langevin et al., 2012; Morrison et al., 2006), as well as physiological changes designed to compensate

for gill pathology. The toxin produced by *P. parvum* is thought to impact fish health through disruption of gill epithelial cell membranes, likely altering osmoregulation and respiration. Although abiotic factors, pollutions such as heavy metals that cause toxicity have also been demonstrated to induce a transcriptome response in immune pathways in rainbow trout gills (Uren Webster et al., 2013; Zeitoun et al., 2014). The tissue response to toxin-producing *P. parvum* might be aligned with responses to environment contaminants, such as heavy metals and infectious agents. Conversely, bivalve response to biotoxins can appear more like an immune response to infection than response to physiological impairment (Galimany et al., 2008), and so the rainbow trout response to *P. parvum* might mirror more closely invasion-type gene expression. .

There is a lack of studies that explore the transcriptome response of fish following exposure to algal toxins. However, the transcriptome response of other aquatic organisms to dinoflagellate toxins, including bivalves and other shellfish, has been explored (Kim et al., 2018). *Argopecten irradians* (Bay Scallops) exposed to oxadaic acid (causative toxin in diarrhoeic shellfish poisoning, produced by *Dinophysi*s and *Prorocentrum* species) demonstrated a transcriptome response involving genes with known detoxification and immune function (Kim et al., 2018); general functional responses documented across a number of bivalve species (Galimany et al., 2008; García-lagunas et al., 2019; Romero-geraldo et al., 2016). These studies identified a number of genes responding to the toxin, including *heat shock protein 70* (HSP70), a marker of infectious disease and pollution exposure in rainbow trout (Castro et al., 2015). A number of detoxifying enzymes involved in the tissue response of Bay Scallops to toxin exposure are also identified, including *cytochrome P450* enzymes and *Nicotinamide adenine dinucleotide phosphate-oxidases (NADPH-oxidases*), associated with reactive oxygen species production (Kim et al., 2018). Harmful phytoplankton impacts on fish, such as damselfish and Nile tilapia, indicate a similar requirement of antioxidant and detoxifying response to phytoplankton biotoxins, although existing studies measure protein production and performed bioassays rather than investigating gene transcription (Marshall et al., 2003; Mazmancı and Çavas, 2009). Although the action of toxins investigated in other studies might be expected to be different to that of *P. parvum* toxins, due to different composition and harmful modes of action, these

studies still provide valuable insight into the mechanisms of toxic effect and clearance in aquatic organisms. Although the immune repertoire of bivalves organisms is very different to fish (Gestal et al., 2008), and mechanisms of response are likely not identical, these studies still represent the best currently available analogues of transcriptomic response to algal biotoxins.

The publications that do exist regarding altered gene expression to stressors in rainbow trout gills focus on infectious disease, pollutants and environmental stressors such as hypoxia. These studies identify various pathways that might also play a role in the gill response to *P. parvum* exposure. Adaptations to hypoxia, for example, includes cessation of high energy demand processes, such as protein synthesis,, locomotion and cell growth/proliferation (Gracey et al., 2001; Ton et al., 2003). Hypoxia is also known to induce lysosomal lipid trafficking and degradation (Van Der Meer et al., 2005), immune-related gene expression and cellular death (Qi et al., 2018)

4.2.3 Investigating the gill transcriptome response to *P. parvum*

This study aimed to investigate alterations in the gill transcriptome following exposure to *P. parvum* in a challenge trial designed to mimic environmental exposure as closely as possible. Results provide an insight not only into impact on gill tissue with varied concentrations of *P. parvum* exposure, but also provide new information regarding the collective activity of *P. parvum* toxins on gill tissue through the specific response mechanisms of tissue. Examination of the biological pathways altered following exposure was performed to explore the tissue response and biological consequences for fish. Through an understanding of mechanisms of toxin clearance, markers of exposure were identified too. Identification of biomarkers of exposure has the potential to be highly useful in environmental monitoring of fish populations and might also enhance our knowledge of fish health in general.

4.3 Methods

4.3.1 Challenge trial and sample collection

Juvenile rainbow trout fingerlings (approx. 10 – 15 g) were acclimated for 14+ days to laboratory facilities, fed a standard commercial fish feed (INICIO Plus 1.5 mm, Biomar) until 2 days pre-exposure. *P. parvum* cells (Kalmar University Culture Collection, strain KAC 39) for use in the challenge trial were cultured as previously described (Andersen et al., 2016) at the Department of Animal Science, Aarhus University, Denmark.

Fish were challenged in a custom-built facility in three groups. Separate treatment groups were exposed to whole cultures of two concentrations (cells / litre of water) of *P. parvum,* determined to be in the exponential growth phase. Groups of rainbow trout were exposed to concentrations designated 'low' (1.5 x 10^4 cells / L) or 'high' concentration (4 x 10^4 cells / L). A third untreated group acted as a normal control (**Figure 4.1**). Toxin production from *P. parvum* cannot be quantified (Rasmussen et al., 2016), however is suggested to be more consistent during uninhibited algal growth (Moran and Ilani, 1974; Shilo, 1981), and so exponential growth phase cells are utilized in an attempt to normalise toxin exposure (Svendsen et al., 2018). Treatment concentrations were determined based on previous work in determining lethal and sub-lethal exposure densities of *P. parvum* exposure (Andersen et al., 2016). Challenge trials were performed at 16 °C and 15 psu salinity. Aeration was adjusted to maintain oxygen saturation at 80%. Notation was made of clinical signs in fish throughout exposure, with monitoring too of oxygen and pH. The study was terminated by euthanasia of fish with immersion in 0.01% benzocaine. All animal procedures were in agreement with the EU Directive 2010/63/EU for animal experiments and performed with permission from the Danish Animal Experiments Inspectorate.

The challenge trial for this work was conducted prior to author involvement in the project. All work including *P. parvum* culture, fish maintenance, handing and challenge was performed by collaborators at Aarhus University, as part of the HABFISH project (http://www.habfish.dk/), without input from the author and separately from the work of

this PhD. All exploration of the transcriptome response, including laboratory work and analysis, was performed by the author, with the instruction in microarray technique of Dr Elzbieta B Krol (EK).

Figure 4.1 Experimental Design

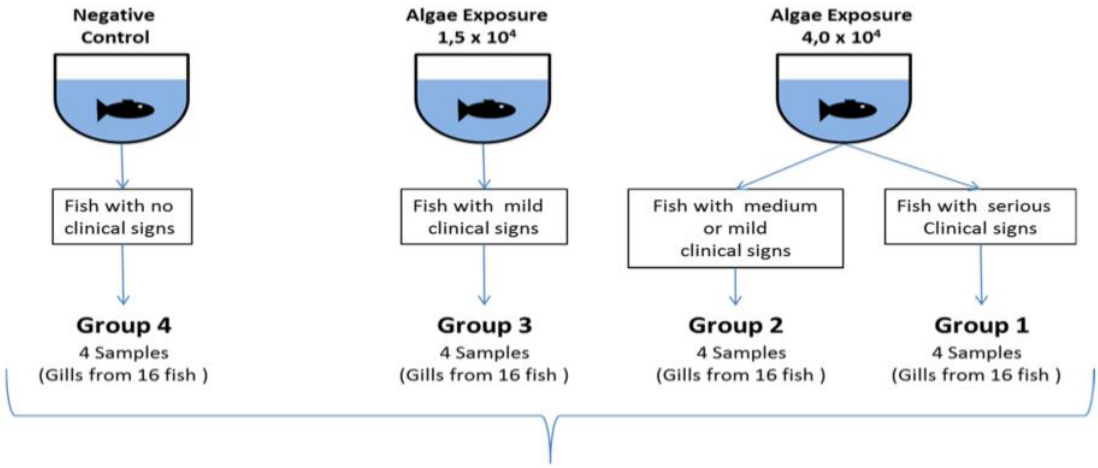


Figure 4.1*: Experimental set up for challenge trial. Fish were exposed to three concentration of P. parvum cells in separate tank facilities before sampling for gill tissue.*

4.3.2 RNA extraction and microarray hybridization

To better understand the biological processes and pathways affected by *P. parvum* infection, microarray analyses were performed on gill tissue collected from fish exposed to *P. parvum* and its biotoxins. RNA was extracted from gill samples as described in Methods (Section 2.3.3) and used to generate labelled aRNA probes for use in a two-colour microarray experiment. These methods represent a summary of experimental procedure, with full details provided in Methods (Section 2.7).

First, RNA extractions were pooled (four individuals to a pool) to generate four biological replications for each clinical presentation group. This pooling of samples was performed based on clinical presentations of mild, moderate and severe along with the control, rather than initial exposure concentration. Despite the original experimental design as three treatment groups, observation of clinical presentation of fish within the

higher concentration treatment during exposure identified two clear divergent responses within fish. It was determined to treat these distinct patterns of clinical response separately, for separate analysis of the gene response of fish apparently less or more severely affected by *P. parvum*. RNA from all fish was then used to generate a common control. aRNA was generated through use of the MessageAmp II aRNA Amplification Kit (Ambion) according to manufacturer's recommendations. Biological replicates were then labelled using Cy3 fluorescent dye (Amersham Mono-reactive Dye Pack, GE Healthcare), and the common controlled labelled with Cy5 (Amersham Mono-reactive Dye Pack, GE Healthcare). Equal concentrations of each biological replicate were then hybridised to 4x44K custom gene expression oligonucleotide 'Trout_imm_v1' microarrays (Agilent design ID: 028918) (Castro et al., 2015). Details of the array platform are available at EBI array express (https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-3401/) under platform accession A-MEXP-2315. Scanning and initial data processing were then performed to generate datasets of global log fold change values for set of treatment group contrasts. Detailed methods for this experiment, from initial pooling to data processing in R using Bioconductor Limma packages (R Core Team, 2018), is provided in Methods (Sections 2.7.1 – 2.7.3)

4.3.3 Computational analysis and figure construction

The gill transcriptome response of fish was assessed by severity of clinical presentation following exposure to 'low' or 'high' concentrations *P. parvum* cells, in an effort to explore the biological functions and pathways of response initiated by *P. parvum* exposure in each group.

Global fold change values were obtained by utilising the mean and standard deviation of values in a simple linear model (Limma package in R) to combine log ratios from microarray features. This analysis generated global expression results, with confidence values, for the following four groups: Control (healthy), Mild, Moderate and Severe. Following *P. parvum* toxin exposure, transcripts were assessed within treated groups using pair-wise differential expression analysis in R to identify the transcripts

differentially expressed relative to the control. Array features were considered differentially expressed if they exceeded the filtering parameters of fold change > 2 and an adjusted Benjamini-Hochberg (BH) p value < 0.01. Array feature sequences were assigned HGNC gene classifications based on data generated by previous work (Castro et al., 2015) where salmon probes were annotated with the best hit to human sequences within the Ensembl database using BlastX. Mapping fish gene sequences to human orthologs has been demonstrated previously (Yadetie et al., 2013) to improve functional analysis through comparison to a well-annotated databases, despite limitations of the mapping due to species differences in gene functions and pathways. Significantly differentially expressed gene (SDEG) lists and various sub-lists were subsequently utilised in downstream analysis.

4.3.3.1 Downstream analysis

Principal component analysis (PCA) was performed on normalised hybridisation datasets of individual arrays, following filtration to remove control features but prior to global fold-change analysis. Analysis was performed using scaled data with the prcomp command inbuilt in R. From this analysis, lists of principal components and associated eigenvalues were identified, ordered by contribution to overall variation. PCA results were visualised using the ggplot and pca3d packages in R. Cluster analysis was performed using array-derived log ratio results (prior to probe filtration for significance and fold change) to obtain k values of feature expression in individual sample datasets. Cluster analysis was performed using the average linkage clustering method with Euclidean distance measurements to cluster results by both row and column for visualisation of the relationships of microarray datasets. Results were visualised using the pheatmap function in R.

To identify possible biological functions of SDEGs, Gene Ontology (GO) and Kyoto Encyclopaedia of Genes and Genomes (KEGG) enrichment analyses were performed using the online tool DAVID 6.8 (https://david.ncifcrf.gov/) (Huang et al., 2009). Use of DAVID 6.8 for enrichment analysis mapped SDEGs to GO and KEGG terms in the database, with results provided lists of enriched GO and KEGG terms for each set of

SDEGs. These were subsequently filtered to only include those processes and pathways with SDEG involvement over 10 genes and a BH modified p value below 0.01, reflective of the level of enrichment within the process. GO annotations were obtained at the GOterm_BP_1 (first level of biological process ontology) and GOterm_BP_FAT (lower levels) of biological process ontology, in keeping with results presented by existing publications (Decker et al., 2012; Król et al., 2016). Various tools were utilised in the analysis of resultant lists of GO terms, including online tool REVIGO (Supek et al., 2011) (http://revigo.irb.hr/) with default settings.

4.4 Results

4.4.1 Pattern of clinical presentation following exposure trial with *Prymesium parvum*

Following exposure, treated fish demonstrated clinical symptoms associated with gill damage and apparent respiratory distress. No clinical symptoms were apparent in individuals within the control group, and no alteration to behaviour was observed during the trial period. Fish within the lower concentration treatment group demonstrated a homogenous response characterised as mild, suffering fewest clinical symptoms in a less pronounced clinical response than those within the higher concentration treatment group. These fish were designated the 'Mild' group. Fish within the higher concentration treatment group demonstrated two distinct patterns of clinical response, with some fish apparently much more severely impacted. On the basis of these clinical presentations, the higher concentration treatment group was subdivided into two groups: moderately and more severely impacted individuals. Fish were assigned to these groups based on their clinical presentation, distinguished as moderate or more severe response. Groups were therefore termed that of 'Moderate' and 'Severe' fish. Clinical presentations at varied concentrations of treatment are summarized in **Table 4.1**.

Table 4.1 Clinical response to *P. parvum*

Treatment Group	Clinical response classification	Clinical response description
High (4 x 10^4 cell / L)	Severe	Moribund fish. Presence in lower portion of tank with generalized lack of movement.
	Moderate	Reduced activity of fish. 'Gasping' behavior and erratic movements.
Low (1.5 x 10^4 cell / L)	Mild	Evidence of irritation, with occasional erratic movements
No treatment	None (Control)	No alteration to behaviour

Table 4.1: *Clinical responses in fish treated with P. parvum, and distinction for differentiation of clinical presentation groups from concentration groups.*

4.4.2 Variance in arrays based on clinical presentation

4.4.2.1 Principal component analysis (PCA)

PCA analysis was performed to determine if a global difference in the transcriptome response could be correlated with varied clinical presentation as well as treatment concentration. Principal component's 1 and 2 (PC1 and PC2) account cumulatively for the greatest variation observable within the dataset, 29.7% in total, and were therefore utilised for appraisal of the data (**Figure 4.2**). Plotting PC1 and PC2 in two dimensions (**Figure 4.2)** demonstrates clear separation of samples along both the X and Y axis. Individual array results are plotted in distinct associated with clinical presentation, with particularly tight clustering of the Moderate group arrays. Dimension one, along the x-axis, separates treated groups from untreated controls, with a right to left pattern of distribution dependent on *P. parvum* concentration exposure. Control samples are observed separately from all treated samples. The treatment group with least observable

variance from the control is the Mild group, followed by Moderate and Severe groups. Arrays representing moderately clinically affected fish are observed in close association with those considered severely impacted along this axis (PC1). The second dimension, along the y-axis (PC2), separates treated samples by observed severity of clinical presentation, with overlap of Moderate and Mild array samples. Inclusion of PC3 (**Figure 4.3**) maximises the cumulative proportion of observable variance to 37.4% and provides additional information regarding sample clustering in three dimensions, resolving the artificial superimposition of groups when data is plotted in only two dimensions. From PCA analysis overall, individual arrays clearly separate by both treatment concentration, and by clinical presentation groups. Sample distribution in PC3 follows largely the order in which hybridised microarrays were washed and read.

Figure 4.2 Principal Component Analysis (PCA)

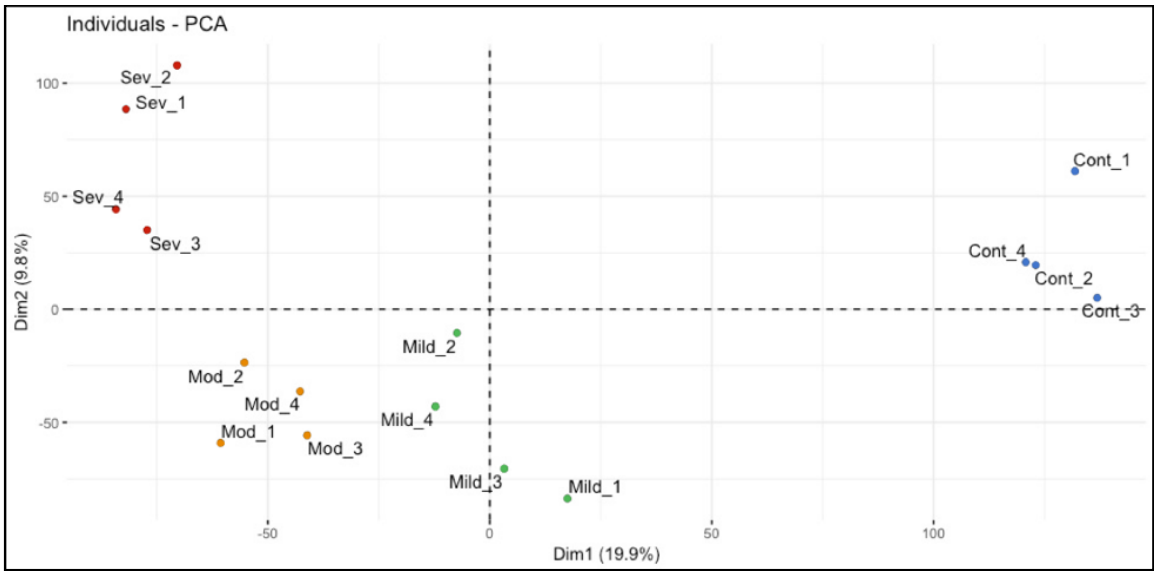


Figure 4.2*: Biplot representation of PCA. Principal components 1 and 2 identified as explaining overall greatest variation within the dataset are plotted in two dimensions along the x and y axis respectively. Arrays are coloured by severity of clinical presentation; blue (control), green (mild), orange (moderate) and red (severe). Clear delineation of results by clinical presentation is observable.*

4.4.2.2 Cluster analysis

Cluster analysis results were illustrated as a heatmap to highlight grouping and separation of samples based on treatments (**Figure 4.4**). Cluster analysis of the expressed transcripts also clearly grouped array results by clinical presentation of fish. Although samples designated as Moderate and Severe were subjected to the same treatment, results illustrated in **Figure 4.4** demonstrate clear variation in transcriptome response between these groups. with Moderate and Mild groups showing closer association from that of the Severe group. A number of distinct clusters were observable within all groups in **Figure 4.4**, representing probes with shared patterns of expression across groups.

Both PCA and cluster analysis reveal a shared response to exposure of *P. parvum* and its biotoxin. Differences in the transcriptome response between treated groups are also observed with both treatment concentration and the severity of clinical symptoms. This supports the decision to analyse groups based on clinical presentation, reflecting the severity of clinical symptoms, so that fish from within the high concentration treatment group are separated into Severe and Moderate groups.

4.4.3 Global gene transcription relative to the control

4.4.3.1 Positive correlation of magnitude and total differential expression with severity of clinical of presentation

The transcriptional response of gill tissue to toxin exposure was assessed by comparing gene expression in exposed groups with the untreated control. Following filtering, analysis identified 3155, 2356 and 1249 probes to be significantly altered between severe, moderate and mild to the control samples respectively. Following removal of redundant (duplicated features) genes were annotated for HGNC identifiers (as described in Section 4.3.3.1 above) were retained so biological interpretations could be made. This results in a total of 1555 significantly differentially expressed genes (SDEG) within the Severe group (914 up and 641 down). 1180 SDEGs (722 up and 458 down)

were identified within the moderate group, and 598 (420 up and 178 down) within the Mild group. Overall, number of SDEGs correspond with severity of clinical symptoms. The Severe group, treated with the highest concentration of *P. parvum* and appearing to suffer the most physiological consequences during exposure, also demonstrated the greatest number of SDEG involvement (**Figure 4.5**). An observable greater magnitude of differential expression with increased severity of clinical presentation was apparent for the majority of shared expression genes too.

When filtering parameters are altered to include only those SDEGs displaying a fold change in excess of 10x relative to the control, these SDEG numbers are greatly reduced. This approach was based on the rationale that genes in this category might be considered particularly responsive to the *P. parvum* treatment (**Figure 4.6**), and facilitates appraisal of a more manageable number of SDEGs, expressed with greatest magnitude of change within different presentation groups. The trend of great overall SDEG involvement in more severely impacted groups is maintained in this reduced list.

Figure 4.3 3-D PCA

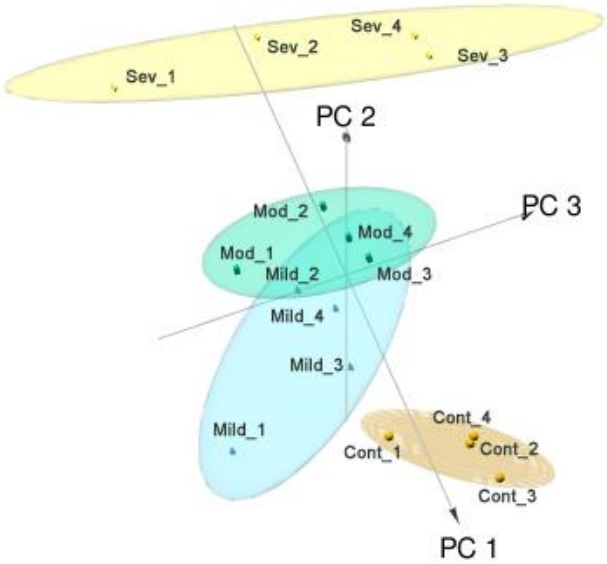


Figure 4.3: *Three-dimensional representation of PCA results. Principal components 1, 2 and 3 are represents to explaining great percentage variation within the dataset. Association of arrays can be observed by severity of clinical presentation, with minimal overlap.*

Figure 4.4 Heatmap of gene expression

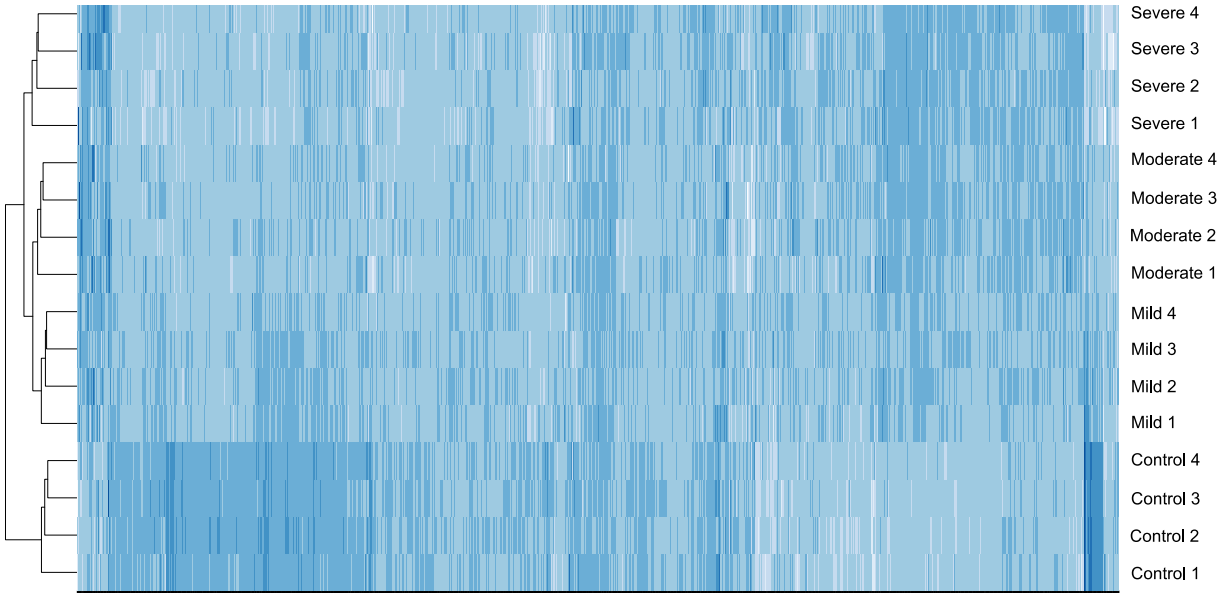


Figure 4.4: *Heatmap demonstrating cluster analysis of all probes from individual arrays using average linked clustering method with Euclidean distance measurements. Clustering can be observed between individual samples (columns), orientating arrays by severity of clinical presentation. Clustering of rows demonstrates shared expression patterns across probes.*

Figure 4.5 Total gene and GO pathway involvement

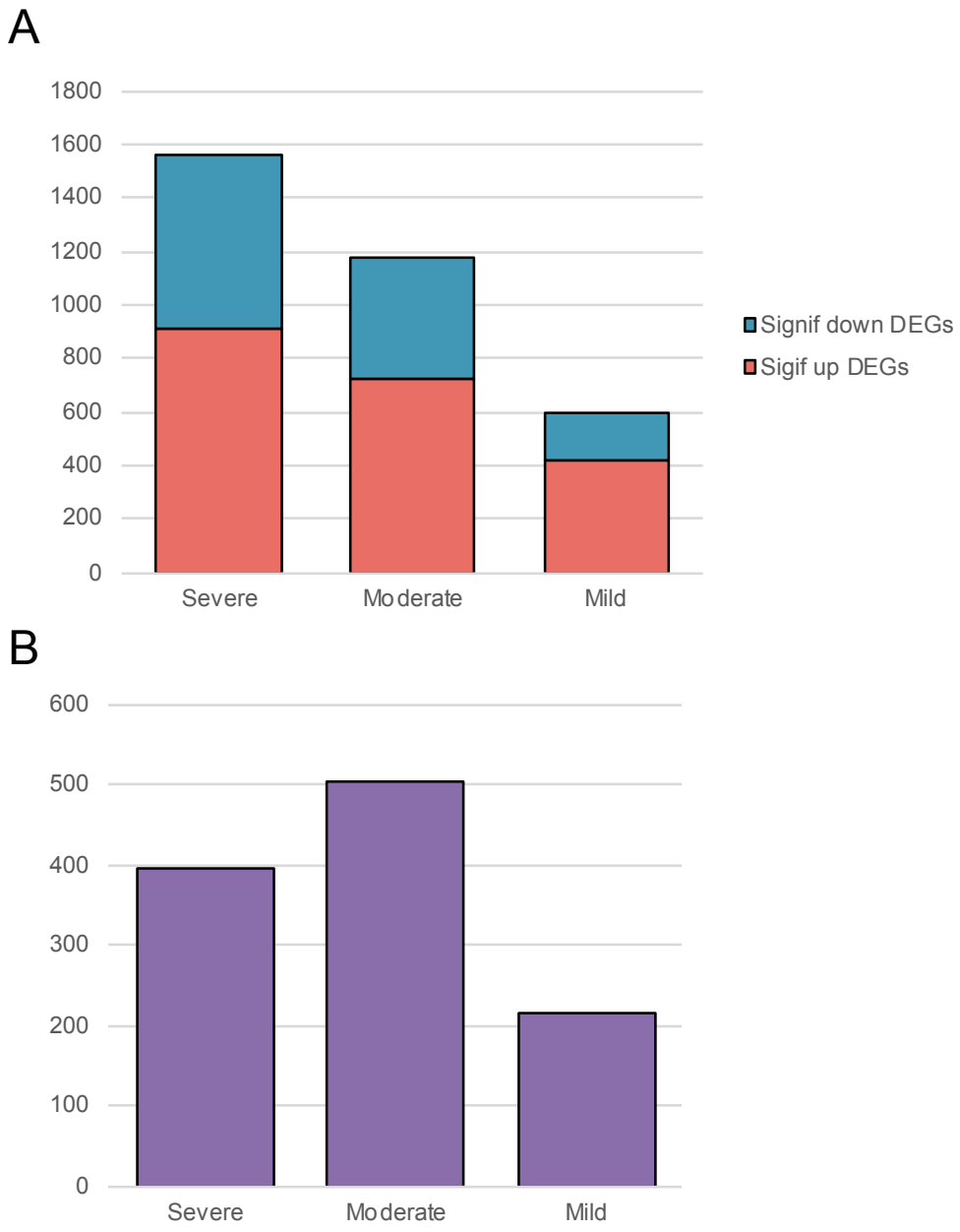


Figure 4.5*: Total SDEG and GO pathway involvement across clinical presentation groups.*
A: Total SDEG involvement within clinical presentation groups relative to the control. Number of SDEG (of both up and down regulated) can be observed to increase with severity of clinical presentation.
B: Total number of significantly enriched GO processes identified from SDEG lists in A.

4.4.3.2 Evidence for a shared response and driving factors of divergent clinical presentation

The majority of SDEGs were common to all clinical presentation groups, with a smaller number unique to individual groups (**Figure 4.7**), demonstrating a conserved general response to *P. parvum* exposure. The Mild group contained few unique SDEGs, with most identified too in other clinical presentation groups. Most of the SDEGs associated with the Moderate group were also differentially expressed in the Severe group too. The majority of unique SDEGs were associated with the Severe group, and of these, 39% were significantly upregulated and 55% downregulated. These results collectively show a similar pattern of SDEG response to *P. parvum* exposure in all three clinical groups, with additional differential expression in the higher concentration treatment groups.

4.4.4 Global gene transcription between groups; significant transcripts with varied clinical presentation

In addition to differential expression relative to the control, clinical groups were also assessed in terms of differential expression relative to each other. This analysis identified genes significantly differentially expressed as a result of different treatments and dependent on clinical symptoms. Comparison of patterns of gene expression between affected groups yielded relatively fewer SDEGs than comparisons relative to the untreated control group, supporting a general response to *P. parvum* exposure shared by all groups. A total of 206 genes were differentially expressed between Severe and Mild groups, but only 10 genes differently expressed between the identically treated groups (Severe and Moderate groups). No genes were identified as significantly differently expressed between Moderate and Mild treatment groups. This analysis was performing using the same criteria for identification of SDEGs (detailed in Section 4.3.3.1) as assessment of treatment groups relative to the control. Full lists of these genes are provided within appendix materials (**Appendix B.1**).

Figure 4.6 Ten-fold change genes

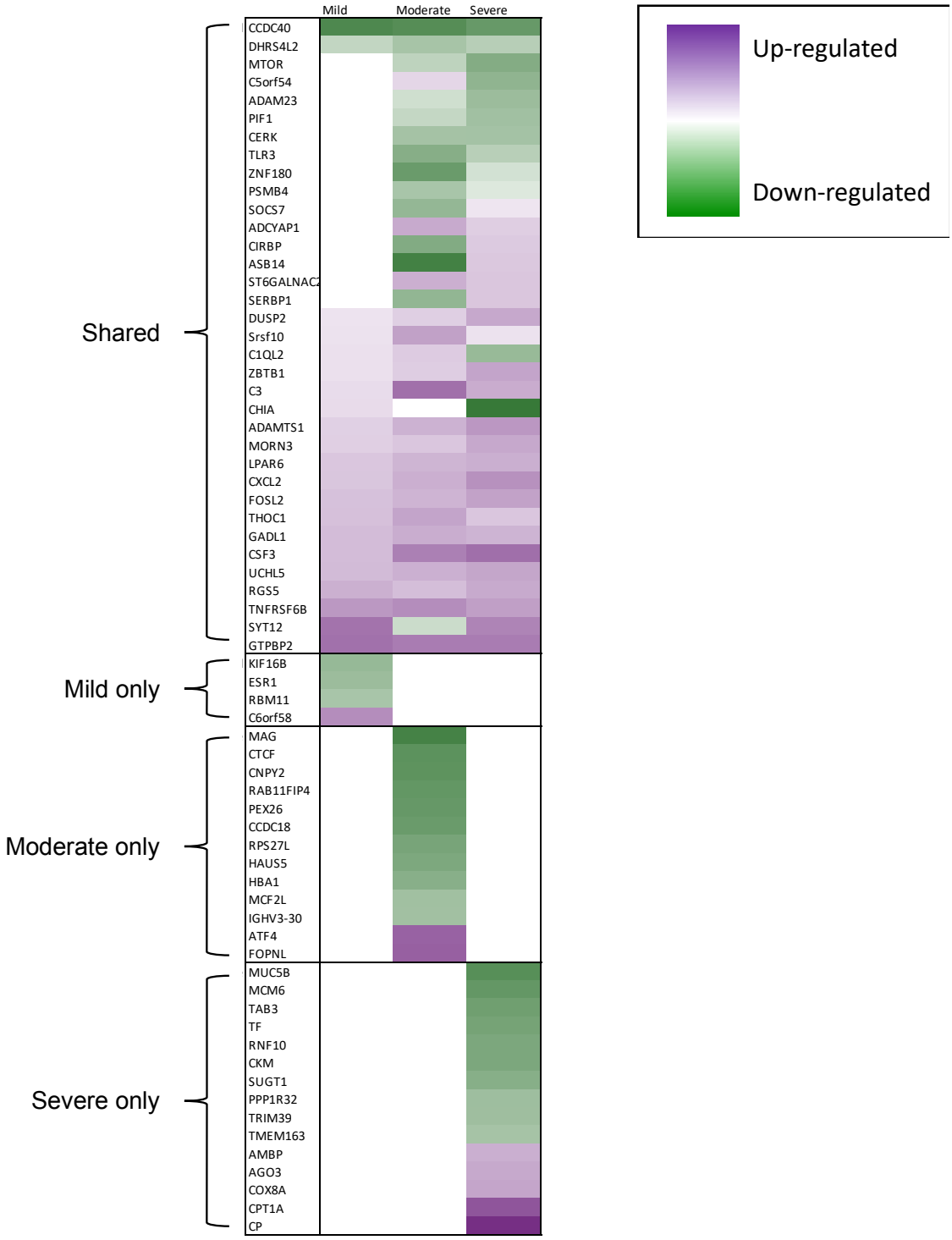


Figure 4.6*: SDEG's identified within presentation groups with a fold change in excess of x10 relative to the control. Shared and uniquely expressed genes are separated to illustrate the shared and divergent patterns of expressional change within groups.*

Figure 4.7 Shared and unique genes

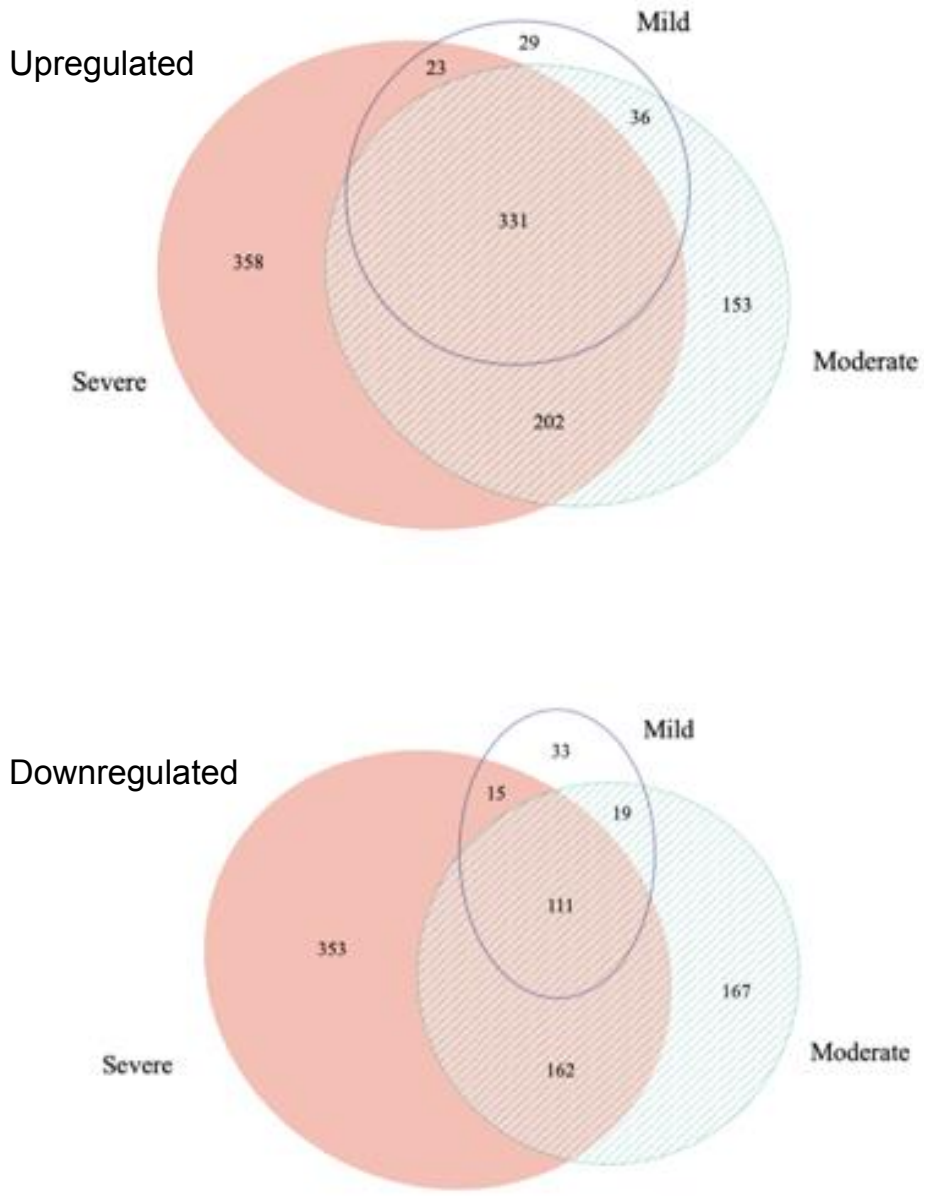


Figure 4.7: *Euler (Venn-type) diagrams demonstrating shared and unique gene features within clinical presentation groups. Uniquely expressed genes are identified mainly within Moderate and Severe groups, with the majority of SDEGs within Mild shared with other presentation groups.*

4.4.5 Biological processes enriched in response *to P. parvum* and biotoxin exposure

Analyses were performed to determine the shared and unique responses within clinical presentation groups, to identify patterns of genes regulated as part of the core response during exposure to biotoxin, and to identify candidate genes potentially underlying differences in clinical presentation in the high concentration treatment group.

4.4.5.1 Shared and unique biological process, molecular function and cellular component enrichment between clinical presentation groups relative to the untreated control

Results obtained mapping SDEGs to GOTERM_BP_1 provided functionality of expression organised by biological process, molecular function and cellular component (Section 4.3.3.1), and a broad overview of the general trends in response to toxin producing *P. parvum* exposure (**Figure 4.8**). Using the described parameters, the majority of differentially expressed genes from each sample group could be assigned to a *biological process* category; Severe - 93.5%, Moderate - 92.6% and Mild - 95%. A total of 96.8% of Severe group SDEGs, 96.1% of Moderate group SDEGs and 97.3% of Mild group SDEGs were assigned to *cellular components* category, and 91.9% of Severe group SDEGs, 91.1% Moderate group SDEGs and 93.4% of Mild group SDEGs were assigned to a *molecular function* category. SDEGs from all groups were associated with the majority of identified enriched GO *biological processes*, with the Severe group containing the greatest number of SDEGs in all instances. A similar outcome was found as regards *molecular functions*: all groups expressed SDEGs associated with the majority of functions observed, and the number and magnitude of change increased with severity of clinical presentation. *Molecular functions* associated exclusively with the Severe group SDEGs include *antioxidant* and *electron carrier activity*. *Biological processes* associated with *detoxification* appeared to be enriched only in the higher concentration treated Moderate and Severe groups.

These results suggest that similar biological process and molecular function are associated with SDEGs from all clinical presentation groups, supporting the suggestion

of a shared response to *P. parvum* exposure. Additional functions and biological processes were associated with SDEGs exclusive to higher concentration groups, perhaps as a consequence of greater *P. parvum* exposure.

GO process analysis for results using parameters described in Section 4.3.3.1 identified notably large lists of lower level biological processes GO terms enriched within each clinical presentation group, despite use of relatively stringent parameters compared to similar previous publications (Król et al., 2016; Uren Webster et al., 2013). For biological process, a total of 395, 503 and 216 GO terms were considered significantly enriched for fish in the Severe, Moderate and Mild groups respectively. (**Figure 4.5**). Within these large lists of enriched GO terms, many terms can be considered functionally overlapping and there are a number of repeated themes across the different groups.

4.4.5.2 GO term enrichment as part of a shared response to P. parvum exposure

Top 25 Significantly Differentially Expressed Terms

The top 25 significantly enriched GO terms were collated for the different clinical presentation groups (**Figure 4.9**). The majority of these enriched processes were significantly enriched in all treatment groups. In the Severe group the most significantly enriched groups were related to cell death and apoptosis. Similar pathways were found enriched within the Moderate group. The top 25 significantly enriched GO terms for the Mild group are of varied function, apparently dominated by signalling and migration. There was overlap in processes across all three clinical groups, with the Moderate group arguably displaying an intermediate profile: with similarities to both the Severe and Mild group results. GO processes enriched in all groups included immunological defence, cell death and metabolism with a clear trend of increasing significance of common enriched terms, and the number of associated SDEGs, with increased concentration of treatment.

Figure 4.8 Broad enriched GO terms

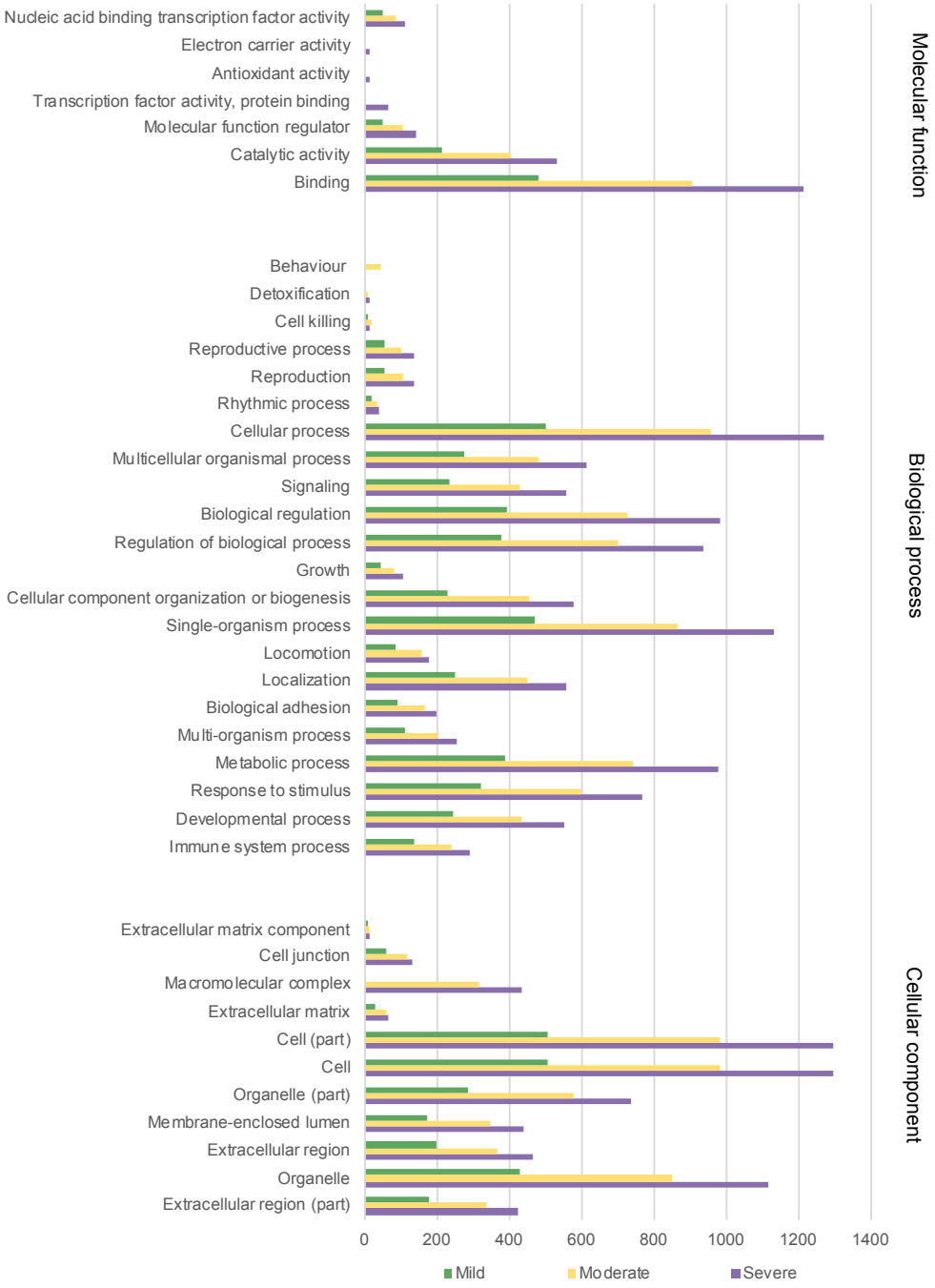


Figure 4.8*: GO terms obtained from analysis of total gene lists against the DAVID GOterm_BP_1 set of classifiers. Numbers represent total SDEG involvement in each process (with many genes likely identified as functioning in multiple processes).*

4.4.5.3 Number of enriched GO terms obtained relative to the control do not mirror trends in total gene expression

GO enrichment analysis of SDEGs identified through differential expression analysis of Severe relative to the Mild group, detailed above (Section 4.4.4 with gene lists provided in **Appendix B.1**) provides insight into the unique functionality of the Severe group. The top 25 enriched GO terms from this comparison (**Table 4.2**) are of varied function, but include processes such as *regulation of cell death, response to oxygen-containing compound, response to oxygen levels* and *response to cytokine.*

Table 4.2 Top enriched GO terms from differential analysis of Mild and Severe

Term	Count	Benjamini
GO:2000026~regulation of multicellular organismal development	47	4.30E-06
GO:0010941~regulation of cell death	41	3.00E-05
GO:0008219~cell death	48	4.26E-05
GO:0042127~regulation of cell proliferation	41	4.87E-05
GO:0009628~response to abiotic stimulus	32	5.74E-05
GO:0006915~apoptotic process	43	9.70E-05
GO:0043588~skin development	15	9.80E-05
GO:0042981~regulation of apoptotic process	37	1.26E-04
GO:0043067~regulation of programmed cell death	37	1.29E-04
GO:0051246~regulation of protein metabolic process	53	1.30E-04
GO:0012501~programmed cell death	44	1.31E-04
GO:0009605~response to external stimulus	47	1.32E-04
GO:0072358~cardiovascular system development	29	1.35E-04
GO:0072359~circulatory system development	29	1.35E-04
GO:0036293~response to decreased oxygen levels	16	1.39E-04
GO:0060548~negative regulation of cell death	28	1.53E-04
GO:0014070~response to organic cyclic compound	28	1.74E-04
GO:1901700~response to oxygen-containing compound	37	1.80E-04
GO:0009725~response to hormone	27	1.81E-04
GO:0034097~response to cytokine	26	1.86E-04
GO:0070482~response to oxygen levels	16	1.99E-04
GO:0009612~response to mechanical stimulus	13	2.40E-04
GO:0001944~vasculature development	22	2.65E-04
GO:0001666~response to hypoxia	15	3.01E-04
GO:0032268~regulation of cellular protein metabolic process	49	3.05E-04

Table 4.2: *Enriched GO terms from analysis of SDEG genes between Severe and Mild groups (genes determines to represent the difference in transcription profile between these groups). The first 25 GO terms (ordered by adjusted p value of significance) are illustrated, including repeated functions in cell death and response to hypoxia.*

Figure 4.9 Top enriched GO terms relative to control

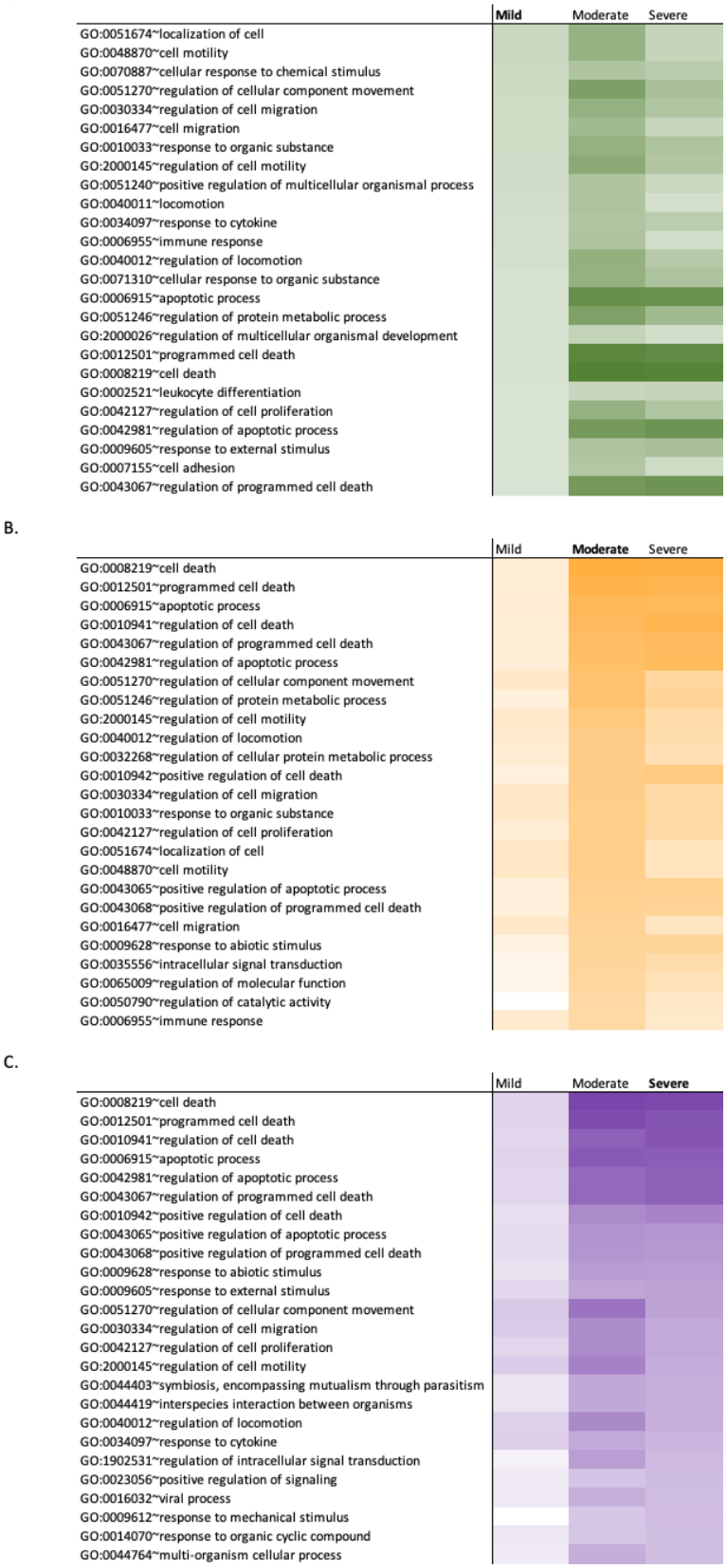


Figure 4.9*: Top 25 enriched GO terms for Mild (A), Moderate (B) and Severe (C) groups, when SDEG are obtained relative to the control.*

Unique GO terms

With regards GO terms identified uniquely within different clinical presentations, a great deal were identified for Severe and Moderate groups. Examples of this include the terms *cellular response to hypoxia*, *cellular response to decreased oxygen levels* and *response to reactive oxygen species* within the Severe group. Many of the GO terms exclusively identified as significantly enriched for Moderate SDEGs have immune-associated function, including *interleukin-8 secretion, regulation of interleukin-12 production, regulation of B cell mediated immunity, regulation of immunoglobulin mediated immune response* and *interleukin-12 production*. Only six GO terms in total were identified uniquely within the Mild group. There included *nitric oxide metabolic process, reactive oxygen species biosynthetic process, reactive nitrogen species metabolic process* and *positive regulation of nitrogen compound metabolic process*. Complete lists of these GO terms are provided in **Appendix B.2.**

REVIGO

Redundancy between GO terms and biological processes, such as seen in the multiple cell death associated GO terms in **Figure 4.9**, can be minimised by use of the program REVIGO, which collapses parent-child relationship and reduces semantic similarity in GO terminology. Use of REVIGO can further inform the grouping of biological processes. Visual summaries of these reduced GO pathway lists were obtained from the program REVIGO, which illustrates relative significance of processes and colour-codes by shared functionality (**Appendix B.3**). For the overall lower number of enriched GO terms within the Mild treatment group, no dominant singular biological response is apparent within REVIGO visualisation. Based on the results of PCA (**Figure 4.2**) the Mild group appears to most closely mimic the 'normal' gill function of experimental fish, as defined by the control. A number of biological processes are clearly enriched within the Mild treatment group, with the majority of these shared with other treatment groups and may be regarded as a 'core' response to *P. parvum* exposure. Results of REVIGO appear highly similar for Severe and Moderate derived GO processes, supporting a shared functionality of response at high concentration treatment. Overall,

these analyses suggest an initial common response to *P. parvum* is supplemented by additional responses in the Moderate and Severe groups, with increased SDEG involvement in a number of responses with shared thematic processes, like cell death.

4.4.6 KEGG pathway enrichment with exposure to *P. parvum*

KEGG pathway analysis highlights a number of pathways of importance in the gill tissue response to *P. parvum* exposure. Inflammation appears to be increased across all clinical presentation groups, with *Tumour Necrosis Factor (TNF*) signalling significantly enriched in all treated fish. A core shared response of *TNF*-signalling genes is apparent in all treated groups, with a general pattern of increased differential expression and additional SDEG involvement with increased severity of presentation (**Figure 4.10**).

TNF is the only KEGG pathway with significant SDEG activation across all three clinical presentation groups. Other pathways, identified for separate clinical presentation groups, include enriched *T cell receptor signalling*, *cytokine-cytokine receptor interaction*, *neurotrophin signalling* and *HIF-1 hypoxia associated signalling* pathways in the Moderate group. Pathways considered enriched within the Severe group include anti-apoptotic *Jak-STAT* and metabolic *FoxO signalling* pathways, as well as *p53 apoptotic signalling* (**Table 4.3**).

Figure 4.10 Signalling pathway

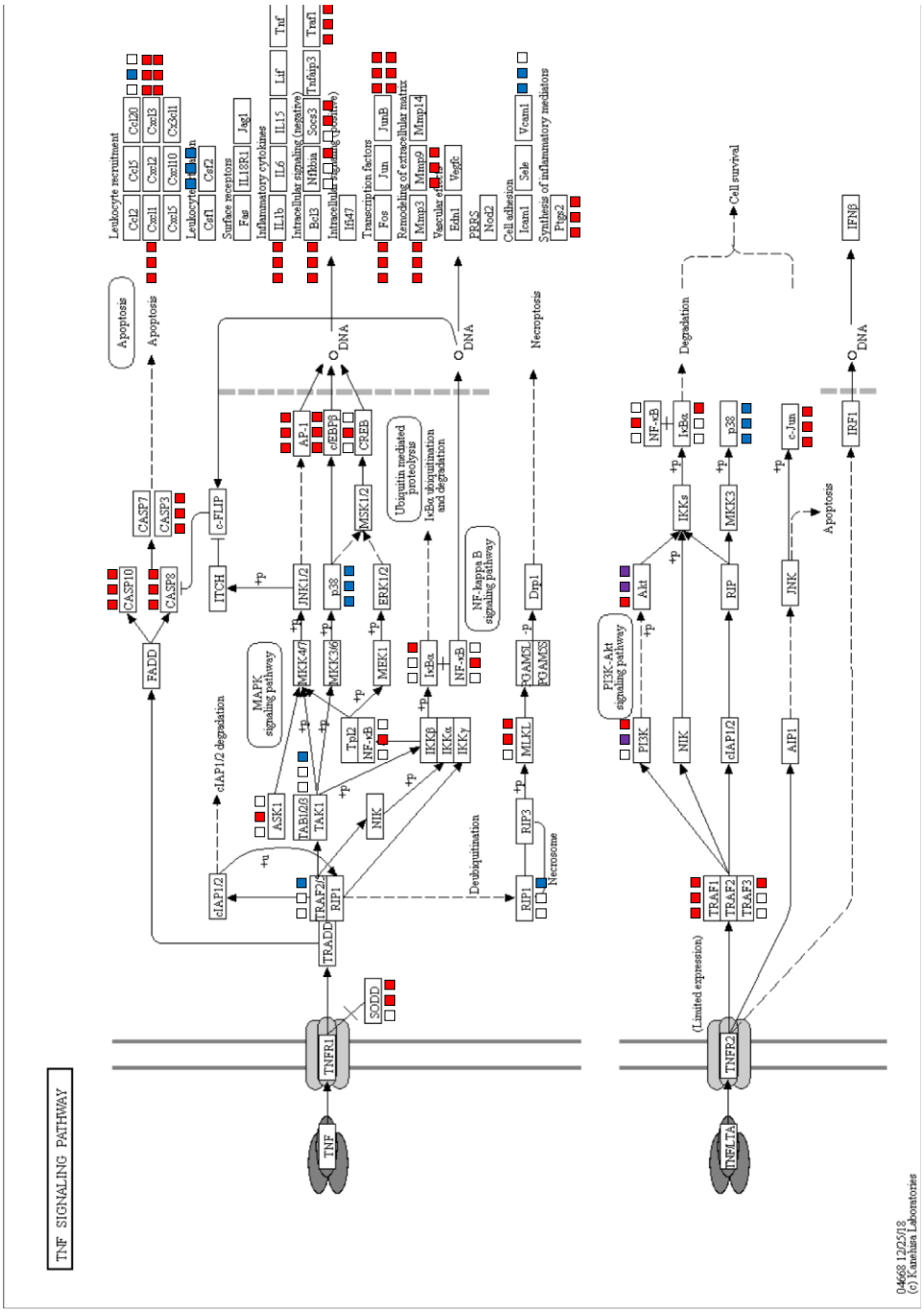


Figure 4.10: *TNF KEGG pathway. Boxes represent, from left to right, Mild, Moderate and Severe clinical presentation groups. Boxes in red represent an upregulation of the associated transcript for that step in pathway progression, and blue represents downregulation of the associated transcript for pathway progression.*

Table 4.3 Enriched KEGG pathways

hsa05134:Legionellosis	20	7.7642E-05
hsa05200:Pathways in cancer	67	0.00048254
hsa04668:TNF signaling pathway	26	0.00159943
hsa04068:FoxO signaling pathway	29	0.00410869
hsa04110:Cell cycle	27	0.00470384
hsa05222:Small cell lung cancer	21	0.00487704
hsa04630:Jak-STAT signaling pathway	30	0.00560351
hsa03320:PPAR signaling pathway	17	0.01665957
hsa04610:Complement and coagulation cascades	17	0.01888129
hsa05145:Toxoplasmosis	24	0.01895014
hsa00270:Cysteine and methionine metabolism	12	0.02009951
hsa01230:Biosynthesis of amino acids	17	0.03215721
hsa04115:p53 signaling pathway	16	0.03342434
hsa05203:Viral carcinogenesis	34	0.04129804

Moderate	Count	Benjamini
hsa04668:TNF signaling pathway	27	5.5665E-06
hsa05134:Legionellosis	19	8.7069E-06
hsa04068:FoxO signaling pathway	27	0.00047281
hsa05200:Pathways in cancer	54	0.00102883
hsa05145:Toxoplasmosis	22	0.00841595
hsa04660:T cell receptor signaling pathway	20	0.00882798
hsa04151:PI3K-Akt signaling pathway	45	0.01162351
hsa05133:Pertussis	16	0.01247591
hsa05222:Small cell lung cancer	17	0.0131794
hsa05161:Hepatitis B	24	0.01340983
hsa04630:Jak-STAT signaling pathway	24	0.01340983
hsa05140:Leishmaniasis	15	0.01504641
hsa04917:Prolactin signaling pathway	15	0.01504641
hsa05152:Tuberculosis	27	0.01618135
hsa04060:Cytokine-cytokine receptor interaction	32	0.01936901
hsa04722:Neurotrophin signaling pathway	20	0.02691712
hsa05220:Chronic myeloid leukemia	14	0.03759787
hsa04620:Toll-like receptor signaling pathway	18	0.03807423
hsa05144:Malaria	11	0.03946362
hsa04066:HIF-1 signaling pathway	17	0.03954575
hsa05211:Renal cell carcinoma	13	0.04027801
hsa05203:Viral carcinogenesis	28	0.04065864
hsa05202:Transcriptional misregulation in cancer	24	0.04293671
hsa03320:PPAR signaling pathway	13	0.04507353
hsa04932:Non-alcoholic fatty liver disease (NAFLD)	22	0.04796093

Mild	Count	Benjamini
hsa05134:Legionellosis	14	4.7397E-05
hsa04668:TNF signaling pathway	18	0.00016425
hsa05152:Tuberculosis	21	0.00314477
hsa05200:Pathways in cancer	33	0.0078678
hsa05140:Leishmaniasis	12	0.00864141
hsa04060:Cytokine-cytokine receptor interaction	22	0.01801183
hsa04976:Bile secretion	11	0.02001974
hsa05222:Small cell lung cancer	12	0.02138918
hsa05133:Pertussis	11	0.02606725

Table 4.3 *– KEGG pathways enriched for SDEG expressed relative to the control in each clinical presentation group. Highlighted pathways were considered statistically significantly upregulated based on the adjusted p value of 0.01. Additional pathways are those meeting 0.05 p value criteria.*

4.4.7 Transcripts modified in response to *P. parvum*

GO analysis identified numerous processes enriched in gill tissue following exposure to *P. parvum*. Many of these processes are shared, but some GO processes and SDEG involvement appears specific to fish treated with higher concentration of exposure. Combining these data with our existing knowledge regarding *P. parvum* toxin exposure, a subset of SDEGs were selected for further discussion.

4.4.7.1 Gene categories affected in tissue response to P. parvum; Toxin clearance and antioxidant response

Existing research on exposure of aquatic organisms to algal toxins suggests an important role for antioxidant and detoxifying mechanisms in the clearance of toxins (De Jesuś Romero-Geraldo et al., 2014; García-lagunas et al., 2019; Romero-geraldo et al., 2016), albeit to different HAB organisms. Broad GO analysis identified enrichment of these biological processes following rainbow trout exposure to *P. parvum*. A number of genes with function in *detoxification* and *response to reactive oxygen species* (ROS) production were considered differentially regulated in this dataset.

Antioxidant-regulating nuclear factor E2 and *Heme oxygenase* with anti-oxidase activity were upregulated across all treated groups. *Heme oxygenase* is expressed during cellular stress with oxygen deprivation and exposure to free radicals (Panchenko et al., 2000), with a proposed function in catalysing the degradation of heme, to prevent deleterious effect to cells. Elevated expression of additional genes with antioxidant products were observed too in the high concentration groups including *Antioxidant-regulating Nuclear Factor-E2-Related Factor*, with a proposed role in the response to oxidative stress (Park et al., 2016). Transcripts for antioxidant enzymes *Catalase, Glutathione peroxidase* and *Fatty acid binding protein 1* were all downregulated significantly within the Severe presentation group

Genes associated with detoxification of toxic compounds and response to DNA damage were also identified as differentially expressed within treated fish, with upregulation of

Ceruloplasmin and *Protein Tyrosine Kinase 2 Beta* within the Severe group, and increases in *Cytochrome p450 family enzymes* and *ABC transporter proteins* across treatment groups. The *cytochrome P450* (*CYP450*) enzymes are a family of proteins related to metabolism of xenobiotics (Schlenk et al., 2008), and can be both induced and inhibited by various toxins in salmonids. Several SDEGs in this family were identified, including *CYP1A1* increased in Severe group. *Smad 3* gene expression was also identified within the Severe group. Upregulation of *ABC transporters* in this study suggests a universal mechanism for these products in toxin removal**.** Again, a different pattern of expression is observed across Severe and Moderate presentation groups, with both unique and shared patterns of gene expression. Differential expression of detoxifying enzymes may reflect the activation of different detoxification pathways in different treatment groups, resulting in differences in observed severity of physiological compromise. The changes in the level of the *Ceruloplasmin* transcript represents the greatest differential expression relative to the control observed in this study: a 399x fold increase in the Severe relative to Control group. *Ceruloplasmin* functions in oxidisation of toxic ferrous to a non-toxic form within tissue (Patel et al., 2002), suggesting a requirement due to ROS accumulation and the need for transformation of the toxic ferrous product to a non-toxic form.

4.4.7.2 Gene categories affected in tissue response to P. parvum; Traumatic stress and inflammation.

A number of GO processes enriched in datasets of treated fish relative to the untreated Control were associated with recovery from traumatic damage. SDEGs expression within these processes included *ST3GAL1* and *ST6GALNAC,* genes previously identified as part of the early healing phase (Sveen et al., 2019), and early ischaemic response genes, *c-fos* and *c-jun* (Dergunova et al., 2018). Upregulation of these transcripts indicated ongoing tissue damage and oxygen deprivation is occurring in the gills of *P. parvum* treated fish.

Cell death signalling is often closely linked to cellular stress and the inflammatory response in rainbow trout, and this study found differential expression of a number of

transcripts for proteins as indicators of cellular stress, including *High-mobility group protein HMGB1, heat shock proteins (hsps), Matrix metallopeptidases* (MMPs) and *Chemokine ligand proteins* alongside various other immune factors.

DNA Damage Inducible Transcript 4 and *Growth arrest and DNA damage* genes were upregulated in response to toxin exposure. The products of these genes respond to DNA damage and play a role in the subsequent repair of DNA. Increased expression of transcripts responsive to DNA damage implies ongoing DNA damage. Greater expressional change of these transcripts seen within the Severe group is suggestive of greater damage within this group, indicating that fish within the Severe presentation group are suffering a greater ROS insult than other clinically presenting groups (Fulda et al., 2010). Increased expression of *DNA Damage Inducible Transcript 4* is associated with the Severe group, suggesting a greater degree of DNA damage. This gene is also identified by differential expression analysis between Severe and Mild groups as significantly differentially expressed, supporting the assumption of increased requirement for the products of this gene in fish within the Severe treatment group.

Previous research in other aquatic organisms reported increased expression of putative immune-related genes (Galimany et al., 2008) in response to algal toxins, and this is mirrored in this study with variable expression of immune responsive transcripts. Observed markers of an inflammatory response included those associated with both the innate and adaptive immune response, such as expression of *cytokine, interferon* and *complement* genes. *Heat shock proteins* appear useful, if non-specific, markers of phytoplankton induced biotoxic stress to rainbow trout gills. Genes associated with *heat shock protein* production, including *Hsp90, Hsp70, Hsp10* and *Hsp2*2, were differentially transcribed relative to the control group for all treatment groups in this study. *Hsp70* in particular has been identified in a number of previous studies as an indicator of toxic stress, as well as important in infective conditions. Expression is noted too of a number of Matrix Metallopeptidase enzymes, including *MMP9* and *MMP13*, thought to be regulators of inflammation (Langevin et al., 2012; Vizoso et al., 2006; Wang et al., 2013). Inflammatory transcripts identified in the *TNF* signalling pathway (**Figure 4.10**) such as *Lymphotoxin beta* appear prevalent in expression of all

groups, with over 10x fold change of transcripts with cytokine function in both higher concentrations treated groups. *Chemokine and cytokine Macrophage inflammatory protein 2-alpha* and *Colony stimulating factor 3* are both highly upregulated. *Lymphotoxin Beta*, *Suppressor of cytokine signalling 3 protein encoding gene* SOC3 and *Interleukin 1 beta* were all immunological transcripts identified as significantly differentially regulated between Severe and Moderate datasets directly (**Appendix B.1**).

Further transcripts associated with pro-inflammatory cytokine expression include those for production of interleukins, of which there were a good deal of SDEGs. *Interleukin 1 Beta*, *Interleukin 11* and *Interleukin 8* were expressed by all treated fish with greatest magnitude in Severe, and *Interleukin 10* was expressed in all groups with greatest magnitude of expression in Moderate. In fact, in contrast to the general pattern of increased magnitude of change with increased severity of clinical presentation, many SDEGs with immune function appear altered with greater magnitude of relative change in the Moderate group. This included complement-associated transcripts such as *C5AR1* and *Complement component 3* (*C3*). Inflammatory mediator *Nitric oxide synthase* (Sharma et al., 2007) was also upregulated with greatest magnitude in Moderate fish, along with *activating transcription factor ATF4* with an expressional change relative to the control in excess of 10-fold. This gene encodes a stress induced factor essential for transcription of key genes for adaptive functions (B'Chir et al., 2013). Further primary cytokine associated expression includes upregulation of as transcript assigned to *Granulocyte colony-stimulating factor (CSF3).*

4.4.7.3 Gene categories affected in tissue response to P. parvum; Physiological disturbance

Hypoxia

Hypoxic-responsive pathways of physiological adaptation were observed as stimulated within gill tissue, with hypoxia-responsive genes generally upregulated following *P. parvum* exposure. *Hypoxia-inducible factor 1 alpha (HIF-1a)* mRNA (an indicator of acute hypoxia in fish) (Pelster and Egg, 2018) was upregulated along with other *HIF-1a*

pathway associated SDEGs. Downstream effects of increased *HIF-1a* signalling, including cardiovascular and metabolic effects, were also observed. These included differential expression of *Vascular endothelial growth factor A* which is associated with proliferation and migration of vascular endothelial cells (Cucina et al., 2003) and the *6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 enzyme* gene transcript *PFKFB3* involved in glycolysis. An essential component of the transcriptional response to hypoxia in fish involves metabolic adaptation via glycolysis, so ATP use mirrors ATP production. KEGG pathway analysis (**Appendix B.4**) suggests the involvement of HIF-*1a* signalling in inducting of glycolysis in *P. parvum* treated fish. Further SDEGs associated with this metabolic adaptation (Yarev et al., 1961) include the upregulation of expression of *fructose-bisphosphate aldolase (ALDOA*) and *Hexokinase-1* genes, for transition from aerobic respiration to anaerobic glycolysis, and the upregulation of *AKT Serine/Threonine Kinase 2* and *Leptin* expression.

Increased expression of glycolytic genes therefore suggests metabolic adaptation, most likely due to the oxygen deprivation experienced by the treated fish. Impaired oxygen delivery resulting from gill damage may further perpetuate tissue trauma, suggested here by changes in expression of early ischaemic response genes, *c-fos* and *c-jun* (Dergunova et al., 2018). Upregulation of these transcripts indicated ongoing tissue damage and oxygen deprivation is occurring in the gills of *P. parvum* treated fish.

Also upregulated were genes associated with metabolic function, including mitochondrial respiration enzyme *cytochrome c oxidase subunit 8A* (*COX8A*) and *carnitine palmitoyl transferase* for fatty acid oxidation, upregulated over 10x within Severe. Smad 3 gene expression, identified in the Severe group only, can be indicative of repair of ROS-induced mitochondrial damage. Variable upregulation of ubiquinol-cytochrome c reductase complex subunit genes as part of mitochondrial respiratory complexes III and V is also identified, suggesting an elevated production of ROS.

Osmoregulation

Upregulation of the osmotic stress response transcript *insulin-like growth factor 1* was seen in tissue from the high concentration treatment groups, as well as *prolactin* expression. Elevated expression of these genes has previously been demonstrated alongside ion-balance regulating factors as part of hyperosmotic adaptation (Evans and Somero, 2008; McCormick, 2001; Sakamoto and McCormick, 2006). Concurrent upregulation of *anion channel cystic fibrosis transmembrane conductance regulator (CFTR)* transcript in the Severe presentation group, for increased NaCl secretion (Bodinier et al., 2009) alongside upregulation of ionic-balance regulating factor *claudin* tight junction encoding protein genes *(CLDN* genes) in all treatment groups was also observed, suggesting an increased requirement for osmotic control in gills.

4.4.7.4 Gene categories affected in tissue response to P. parvum; Cell death and apoptosis

Previously studies note the cytotoxic consequences of *P. parvum* exposure, and it is apparent from GO analysis that altered transcription of cell-death-associated factors is a major element of the tissue response to *P. parvum*. Pathways in apoptosis were therefore of interest, and through KEGG analysis, pathways with function in signalling for cell death were examined in more detail to determine SDEGs involvement, and potential consequences of expression. *p53 signalling* and major inflammatory response driver, *TNF signalling*, were found to be significantly enriched. Upregulation of a number of *Tumour Necrosis Factor* and *Caspase* transcripts was identified. Within the *TNF* signalling pathway, there are SDEGs associated with necroptosis as well as apoptosis (**Figure 4.10**). Expression of the necrosis associated genes *Receptor interacting protein (RIP)* and *Mixed lineage kinase domain-like protein (MLKL)* was associated with the high concentration exposure groups. Transcripts associated with pyroptosis as elements of *Nod-like receptor signalling* were also found to be upregulated in this study, particularly in the moderate group. Autophagy however appears not to be a dominant mechanism of cell death in *P. parvum* exposed gills, with *autophagy related 16-like 1 (ATG16L1)* and *autophagy-related gene 7 (ATG7*)

downregulated in the higher concentration groups. Increased expression of *thrombospondin 2,* with activity in both the induction of apoptosis and suppression of angiogenesis (Mirochnik et al., 2008; Noh et al., 2003), was identified. Results of differential expression analysis between Severe and Moderate groups directly identified a small number of transcripts with significantly greater expression within the Moderate group relative to Severe, and they included upregulation by Moderate of *transcription factor LMO7* and the connexin protein product of *GJA3* and *RALB*, associated with cell proliferation and survival. Relative upregulation by Severe were SDEGs ultimately associated with inflammation.

4.4.7.5 Gene function in tissue response to P. parvum; Signalling in mucus production

A number of genes identified as potential drivers of variation between Moderate and Severe group transcriptome responses have function in mucin production and the Sonic Hedgehog pathway. For example, the gene exhibiting the greatest relative decrease in the Severe group was *mucin 5B*, suggesting downstream a reduced production of mucin glycoprotein. Both *Mucin 5B* and *Mucin 2* were identified as significantly differentially expressed through comparison of Severe relative to Mild transcriptomes (**Appendix B.1**), suggesting a generalised downregulation of production of large secreted gel-forming mucins (SGFM) with increased *P. parvum* exposure (Malachowicz et al., 2017)

4.4.7.6 Gene function in tissue response to P. parvum; Haemolytic and neurotoxic consequences

Possible neurological effects of *P. parvum* toxin exposure might results from downregulation in expression of genes associated with neurological signalling, including an over 10x change in *guanine nucleotide exchange factor (MCF2L),* for stabilization of glutamatergic synapses (Hayashi et al., 2013; Ko et al., 2016). Expression of *Myelin associated glycoprotein (MAG)* required for maintenance of normal axon myelination was also greatly downregulated within the Moderate group. *Heme oxygenase* expression across groups a marker for erythrocyte destruction in

haemolysis might suggest the presence of products of erythrocyte lysis within gills (Rokushima et al., 2007) and upregulation of a number of *coagulation factors* and receptors suggested an increased requirement for clotting factors with haemolysis induced impaired clotting times (Hernaningsih and Akualing, 2017). Of the SDEGs identified directly between Severe and Moderate (**Appendix B.1**), two were associated with function in angiogenesis and clotting; *Thrombospondin 2* and *Lymphatic Vessel Endothelial Hyaluronan Receptor 1*.

4.5 Discussion

This research investigated the gill transcriptome response of juvenile rainbow trout following exposure to toxin producing *P. parvum* cells. It is the first study of its kind and provides initial insight into the action of mixed *P. parvum* biotoxin exposure, and the mechanisms of tissue response, as measured by relative transcriptomic change within gill tissue. Although many studies have investigated changes in gene transcription with other toxic or harmful insults, none so far have explored the impact of HAB biotoxins.

Results indicate that, mechanistically, the gill response observed was shared across treatment groups, with increased exposure resulting in increased effect (although a divergence in clinical presentation and SDEG gene profiles was observed between fish at the high treatment concentration, that warranted exploration). Overall, these results provide valuable insight into not only the biological consequences of *P. parvum* exposure, but also the mechanisms through which these phytoplankton elicit gill damage, and how fish respond. By exploring the SDEGs expressed in gill tissue following exposure of fish to this HAB phytoplankton, results demonstrate a variety of consequences of exposure.

4.5.1 Study design

The challenge conditions utilised for data collection were selected to mimic as closely as possible the exposure of fish in the wild or aquaculture facilities to toxin producing blooms, to obtain a representative profile of the transcriptome response. Much of the toxic effect of *P. parvum* appears to be through direct contact of cells with fish gills, with at least some toxic proteins of this phytoplankton apparently present within the cell wall (Andersen et al., 2016), a factor unaccounted for in many toxicological studies. Toxin exposure appears to act primarily on the gills, impairing the functionality and polarisation of gill epithelial cells (Ulitzur and Shilo, 1966), so gill tissue was determined to be the most biologically interesting source of RNA for assessing the impact of toxin exposure. The knowledge that gills are sensitive to and often one of the

first organs to demonstrate change following exposure to environmental toxins (due to their large surface area and contact with the water (Castro et al., 2018)) further affirmed this choice.

From results, a clear response to exposure can be observed in *P. parvum* treated fish, both clinically and in the pattern of transcriptome response. Following differential expression analysis relative to the control, it is apparent that a shared transcriptome response (or 'core' response) to *P. parvum* is shared by all treated fish and exemplified by the Mild group. GO and KEGG analysis provided insight into the biological processes of this transcriptome response and focused downstream investigated of SDEGs. Exploration of the function of these genes answered the original question regarding the biological response to toxin producing *P. parvum.* Also of interest was the observed variation in clinical presentation between identically treated fish in the high concentration exposure. Separate treatment of fish considered severely or only moderately impacted by exposure demonstrated clear differences in gene expression between groups. SDEG and patterns of expression between these treatment groups was therefore of interest as possible explanatory variables in the divergent clinical presentations observed. However, following assessment of the function of these genes, function of responses were determined to be shared, albeit with different focus of expression. Moderate fish appear to demonstrate a response more in the functions of inflammation and in clearance of toxins, whereas Severe group fish appear to express more SDEG with function in cell death.

4.5.2 Harmful consequences of *P. parvum* exposure

Analysis identified genes with function in a number of compensatory mechanism and consequences following exposure of fish to *P. parvum*.

Osmotic and hypoxic stress

Many transcripts associated with hypoxia and osmotic stress were identified in fish, which alongside the upregulated expression of genes with function in response to

damage and repair, suggests a role for *P. parvum* in gill trauma. Previous research has noted that gill function in gas exchange is reduced following *P. parvum* toxin exposure (Svendsen et al., 2018), with a proposed impact through disruption of membrane permeability, either through direct lysis of epithelial cells (Yarev and Hestrin, 1961) or altered membrane potential and ion balance (Manning and La Claire, 2010). Apparent activation of compensatory mechanisms as part of the transcription response certainly supports the theory that *P. parvum* stimulated oxygen deprivation, and seeing as tank oxygen concentration were experimentally controlled, this effect cannot be attributed to altered environmental availability. Expression of various transcripts with function in response to trauma and tissue repair certainly support the theory of tissue damage, with *HIF-1a pathway* (**Appendix B.4**) and hyperosmotic adaption factors like P*rolactin* and *Insulin-like growth factor* acting in response to maintain gill homeostasis, through adaptive change and increase ion retention on osmoregulatory surfaces (Evans and Somero, 2008; McCormick, 2001; Sakamoto and McCormick, 2006). Compensatory mechanisms such as these counteract the negative physiological consequences of *P. parvum* exposure, and although it does not appear from fish survival or transcription results as though homeostasis has been lost, for total decompensation in fish, negative consequences of oxygen deprivation were observed in the form of early ischaemic response genes, *c-fos* and *c-jun* (Dergunova et al., 2018).

Cell death

Previous cell culture studies in fish cell lines note the cytotoxic action of mixed *P. parvum* toxin components, demonstrated to cause a cell swelling and lysis response highly similar to the stages now described of necrosis (Dafni and Shilo, 1966). A full understanding of what factors dictate cell fate in programmed or unprogrammed cell death is yet to be determined by the scientific community, however it is known that necrotic cell death can be activated by serine proteases in response to membrane disarrangement (Dong et al., 1997; Warny et al., 2000), whereas necroptosis and apoptotic cell death are controlled by gene expression. Apoptosis is a recognised outcome as part of the gill tissue response to hypoxic stress and toxin exposure (AnvariFar et al., 2018), and necroptosis has been previously demonstrated in rainbow

trout cell lines in response to Cadmium exposure (Krumschnabel et al., 2010). Execution of necrosis in a programmed manner by cells is thought to occur in situations where cell lysis is preferable to apoptosis, such as in activation-induced death of primary T lymphocytes (Holler et al., 2000). Signalling in necroptosis is also known to result in an enhanced inflammatory response; like necrosis, necroptosis results in release of Damage-Associated Molecular Patterns such as *high-mobility group protein*s (*HMG*) and *heat shock proteins (hsps)* for promotion of an inflammatory response (Dagenais et al., 2014).

Based on the existing literature and the SDEGs identified in this study, it seems possible that the cell death documented previously with exposure to this phytoplankton is through initiation of apoptosis, with necroptosis-associated cell death too at higher concentrations of exposure. Reactive oxygen species (ROS) (Palomba et al., 1999) and the products of oxidative damage are documented as initiating necroptosis and 'necrosis-type' reactions in mice (Dhuriya and Sharma, 2018; Tashiro et al., 1999), so it therefore seems possible that necroptosis observed in these rainbow trout is due to the products of ROS-induced or toxic protein-induced damage. Necroptosis has been demonstrated to be important in the response to bacterial and viral infections in vertebrates (Goodall et al., 2016) and the results of this study demonstrate its involvement too in the gill response to algal biotoxin exposure. The available literature on cell death suggests oxidative stress elicits an apoptotic response only whilst cells maintain their reducing capacity against ROS. Excessive ROS accumulation then disrupts homeostasis and necrotic-type death instead occurs (Proskuryakov et al., 2003). This would seem possibly to explain the response to *P. parvum* of rainbow trout, where higher concentration treated fish exhibit increased cell death due to greater ROS accumulation and associated damage.

4.5.3 Tissue response to *P. parvum* derived toxins

Although little is known about the consequences of *P. parvum* in fish, exposure of damselfish to *C. marina* results in gill dysfunction through a number of proposed mechanisms, including osmoregulatory compromise through chloride cell disruption,

breakdown of gill membranes with associated reduction in respiratory function, and absorption of toxins and superoxide into the blood stream (Marshall et al., 2003). The toxins of *P. parvum* are many, and as yet not chemically characterised, although modern research has provided some insight; free fatty acids (including stearic acid and an unknown highly labile ichthyotoxic substance) (Henrikson et al., 2010; Marshall et al., 2003), reactive oxygen species (ROS) (Diaz and Plummer, 2018; Marshall et al., 2003) and the prymnesin toxins (Binzer et al., 2019) have all been demonstrated as components of *P. parvum* toxin.

Detoxifying enzymes

Several genes associated with documented function in response to xenobiotics were observed to be upregulated, particularly by fish with the Severe group. This included *cytochrome P450 (CYP450)* genes, documented as important the breakdown of xenobiotics as part of detoxification in fish (Schlenk et al., 2008), and therefore potentially of use in biotransformation of compounds for elimination of algal toxins too. Detoxification enzymes are already known to play an important role in Rainbow trout for removal of toxic substances such as drugs, heavy metals and toxins like fertilisers and pesticides (Petrivalksy et al., 1997). Oxadaic acid exposure in Bay Scallops has been observed to promote differential expression of a number of enzymes within the CYP450 family (Kim et al., 2018), suggesting these enzymes participate in detoxification of protein toxins through transformation of phytoplankton-derived liposoluble toxic chemicals (Liu et al., 2014). Other transcripts with detoxifying-associated function identified included *ATP-binding cassette (ABC) transporter* genes (Kim et al., 2018), proteins responsible for a multi-xenobiotic resistance phenotype in *Perna viridis* and *Mytilus galloprovincialis* (Asian green and Mediterranean mussels) (Prego-Faraldo et al., 2018). *ABC transporters* are thought to act in toxin sequestration, preventing toxin exposure of vulnerable protein and DNA targets in these bivalves (Huang et al., 2015). Obviously, rainbow trout are very different study organisms, and exposed to different phytoplankton in this study, however, differential expression of detoxifying genes appears a common feature of transcriptome response to algal exposure across these toxicology studies.

In addition to lipid peroxidation and DNA damage, toxic moieties of phytoplankton have been demonstrated to impact the reduction of iron through action of ROS (Gonzalez-Davila et al., 1995; K. Wang et al., 2017). Superoxide and hydrogen peroxide free radicals produced in iron (Fe) depleted environments by phytoplankton transform Fe into the more easily absorbed ferrous form, in a presumptive effort for iron acquisition (K. Wang et al., 2017). The ferrous product of this is however toxic within fish gill tissue (Cadmus et al., 2018). *Ceruloplasmin* functions in oxidisation of toxic ferrous to a non-toxic form within tissue (Patel et al., 2002), suggesting a requirement due to ROS accumulation and the need for transformation of the toxic ferrous product to a non-toxic form, for removal of the toxic ferrous product and prevention of further toxicity. Downregulation of blood plasma glycoprotein *transferrin* and *transforming growth factor-activated beta activated kinase 1 MAP3K7-binding protein 3 (TAB3)* suggest too a lack of available free iron due to ROS toxic effects. Whilst ROS is understood to be a part of *P. parvum* toxin components, additional ROS accumulation within the Severe group might have contributed to a worsened toxic effect. Ferrous accumulation within gill tissue appears therefore to be a feature of high concentration *P. parvum* exposure with aggravated ROS impact. Accumulation of toxic iron products in gill tissue would further exacerbate oxidative stress prompting expression of *ceruloplasmin* (Bresgen and Eckl, 2015). *Ceruloplasmin* represents the gene with highest fold change of differential expression relative to the control obtained in this study, observed exclusively within the Severe group with a 399x fold increase. It appears then that one of the major driving factors in divergence in clinical presentation between Severe and Moderate groups is the overall ROS exposure and associated oxidative stress.

Antioxidants and response to reactive oxygen species

ROS are considered an important factor in phytoplankton exposure induced damage in many aquatic organisms (Dorantes-Aranda et al., 2015; Kim et al., 2018), and results of this research support and apparent effect too in rainbow trout exposed to *P. parvum*. A number of antioxidant enzymes appear upregulated, particularly in higher concentration treated fish, for detoxification of ROS to non-reactive molecules and protection of the

organism. *Heme oxygenase* for example, is upregulated in all treated fish, with a function during cellular stress with oxygen deprivation and exposure to free radicals in catalysing the degradation of heme, to prevent deleterious oxidative effect to cells from heme accumulation (Panchenko et al., 2000). A number of antioxidant enzyme genes also appear downregulated. This could have a potentially exacerbating effect on ROS impact (Wang et al., 2015) and seems counter-intuitive to the response to ROS toxicity in gill tissue, where an increase in antioxidant expression would ordinarily provide a protective function (Meilhac et al., 2000). Downregulated expression of detoxifying enzymes has however been previously documented as part of response to ROS (Venkatesan et al., 2006), including in teleosts (Jung et al., 2018). It appears therefore that downregulation of SDEGs for production of *Catalase, Glutathione peroxidase* and *Fatty acid binding protein 1* with antioxidant-activity is a feature of high *P. parvum* toxic insult with ROS exposure. Suppression of antioxidant activity is hypothesised to be an important trigger in the alteration of cell activity from apoptosis to necrotic cell death (Lee and Shacter, 2000; Troyano et al., 2001), a function that may have some use too in toxin clearance.

Although the toxic components of *P. parvum* differ from that of *C. marina*, production of ROS is clearly a shared feature of toxicity in these phytoplankton species, both of which are associated with direct fish kills (Ulitzur and Shilo, 1966). Overall, these results support the conclusion of an accumulation of free radicals with increased severity of clinical presentation, and an action of antioxidant and detoxifying response within gill tissue in response to the toxic effect of ROS and protein components of *P. parvum* toxin. As antioxidant response in a clinical setting is known to occur rapidly, and this study demonstrates the differential expression of potential gene markers in rainbow trout, useful perhaps in profiling acute *P. parvum* toxin exposure.

Immune expression

In addition to ROS and hypoxia-associated cellular markers of stress, numerous markers of an inflammatory response were identified from the results of transcriptome analysis in this study. These included upregulations of transcripts commonly observed in cellular

stress, such as *Heat shock proteins* (*Hsps*), *Matrix metallopeptidases* (MMPs) and *Chemokine ligand* proteins. *Hsps* have been used previously as indicators of environmental pollution in Rainbow trout (Ferreira et al., 2017), and as indicators of toxic impact of phytoplankton in bivalves (Kim et al., 2018). *Heat shock proteins* therefore appear to be a useful, if non-specific, marker of phytoplankton-induced biotoxic stress in Rainbow trout gills. Other SDEG as part of an immune response have shared functionality in innate and adaptive systems. Overall, an induction of inflammation is apparent from the dataset.

Gill surface proteins

Lectins from fish are thought to stimulate superoxide production in *C. marina* (Oda et al., 1998), a factor that may be important too in exposure of rainbow trout to *P. parvum*. Lectin encoding genes are observable as upregulated particularly within the Severe presentation group, a pattern of expression apparently also important role in the pathogenesis of AGD in Atlantic salmon (Morrison et al., 2006). Although insufficient information is currently available, it appears as though lectins within gill tissue and as part of the mucus layer may act too in exacerbation of *P. parvum* ROS activity. If this is the case, the greater severity of clinical symptoms and larger response to ROS in Severe fish might be explained by greater reactive oxygen species exposure and impact.

The gene downregulated with greatest magnitude within the Severe group, *mucin 5B* (identified as well as significantly differentially regulated between Severe and Mild groups*)*, is associated with production of the mucus layer of gill tissues This gene acts in expression of large secreted gel- forming mucins (Malachowicz et al., 2017). Gills traumatised by AGD or other mixed infectious conditions demonstrate a proportional increase in mucus producing goblet cells as part of their morphological adaptation to disease (Peyghan and Powell, 2006), and recent research regarding *Mucin 2* and *Mucin 5* notes altered expression of these genes in association with AGD infection (Marcos-López et al., 2018; Morrison et al., 2006). Rainbow trout are known to respond to gill damage from environmental contaminants with increased mucus production (Ferguson et al., 1992), as mucus contains immunological factors that can assist in protecting gills

from harm (Koshio et al., 2016) and a thicker layer of which affords greater protection to gill tissue. However, a thick layer of mucus can also inhibit respiration. Decreased expression of *mucin 5B* might reflect a number of changes within gill tissue, including a physiology response to improve oxygen uptake in gills experiencing hypoxic stress, or merely as a function of gill remodelling, with proportionally reduced goblet cell numbers as chloride cells increase with osmotic stress. Certainly, this gill response to *P. parvum* is divergent from noted responses to infectious disease such as AGD. Rainbow trout are known to respond to gill damage from environmental contaminants too with increased mucus production, so downregulation appears potentially a maladaptive response (Ferguson et al., 1992)

The roles of *Inositol-Tetrakisphosphate 1-Kinase (ITPK1)*, upregulated in the Severe group, and *FOP-Related Protei*n (*FOPNL/FOP20)*, upregulated with x10 magnitude only in the Moderate group, is unclear. Despite their association in human disease with mucus production, chloride channel function and in respiratory diseases such as cystic fibrosis, there exists no published research regarding the action of these genes in teleosts. The expression of these genes in humans would suggest a role in cilia formation, and although there are no cilia present on the surface of gill cells, they do perhaps have action in some modification of the gill surface. (Chamberlain et al., 2007; Sarmah and Wente, 2010; Sedjaï et al., 2010). Gill remodelling in response to stressors such as hypoxia is a well-established field of study (Blair et al., 2016) and the expression of these transcripts might represent an expressional activity towards that end.

4.5.4 The transcriptome response to *P. parvum*; harmful organism or environmental toxin?

The activity of *P. parvum* toxins on a molecular level is still unknown, however results of this study assist in informing the topic, and that of the cellular response to toxin exposure. Previous work in rainbow trout has identified both shared and unique markers of exposure to various toxins (Hook et al., 2006), assisting in identification of specific modes of action and potential markers for specific contaminant exposure. In addition to markers of DNA damage that suggest a genotoxic effect of *P. parvum* exposure,

detoxifying factors and antioxidant elements of the *P. parvum* response appear similar to toxicological response in gills to chemicals, heavy metals and environmental pollutants. Interestingly though, many environmental toxins are documented as inducing immunosuppression in fish during exposure, the opposite of which is observed with *P. parvum* exposure. The cell stress response as part of *P. parvum* exposure appears then more like an infections condition, however the signalling for mucus production is then divergent from gill disease such as AGD, and necroptosis as a result of ROS exposure it not a commonly observed sequelae of infectious conditions in gills. The overall transcriptome response of rainbow trout gills then seems divergent from both infectious and environmental causes, sitting somewhere in the middle. Results then have the potential for further exploration for specific biomarkers of P. parvum exposure. Results are largely in agreement though with previous conclusions regarding the activity of algal toxins, albeit in bivalves, that report an increase of inflammatory transcripts, rather than the suppression that commonly occurs with heavy metal or effluent toxicity.

4.5.5 Evidence of a shared 'core' response to *P. parvum* exposure

Based on clinical presentation, it was presumed that fish with the lower concentration treatment group would exhibit a less divergent transcriptome response. Results, of PCA and correlated magnitude of change in SDEGs with severity of presentation support this conclusion. The majority of SDEG identified within the Mild group are also shared with both high concentration treatment groups (**Figure 4.7**), indicating that a shared response with specific gene involvement occurs across *P. parvum* treatments (**Figure 4.11**). This 'core' transcriptome response (exemplified by Mild group expression) appears to include tissue transcriptomic activity in response to *P. parvum*, including with significant enrichment of GO terms with function as part of inflammation and the immune response, as well as cell death via *TNF*-signalling (**Figure 4.10**).

Figure 4.11 Shared tissue response to P. parvum exposure

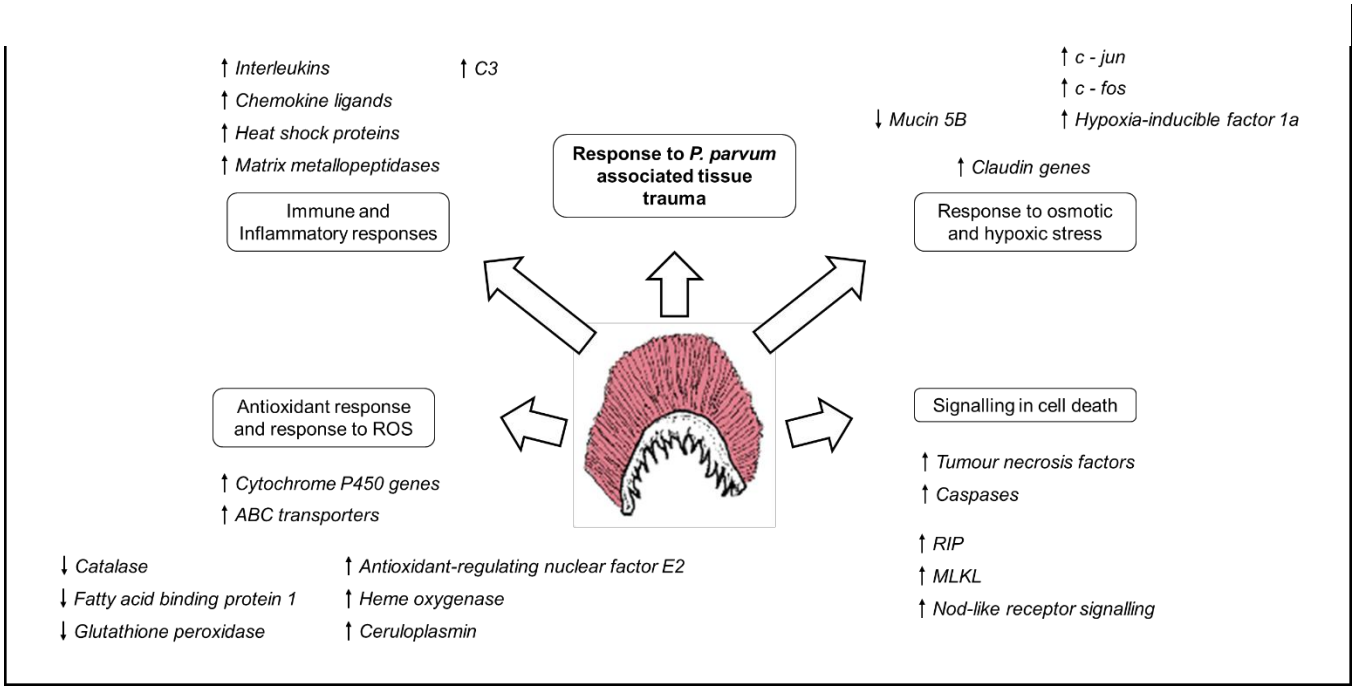


***Figure 4.11**: Core gill responses to P. parvum exposure, including general tissue functions, gene groups and specific genetic expression.*

4.5.6 Dose-effect of *P. parvum* exposure

The action of *P. parvum* in induction of hypoxia response and osmoregulatory factors in fish within the Mild group was notably less than those treated with higher cell concentrations. Fewer SDEG were identified as part of the physiological response mechanisms to these stresses, and so it seems clear that the less severe clinical symptoms observed in Mild fish were due to a lower overall insult, with less functional impairment. A higher concentration treatment appears then to illicit increased oxygen deprivation and osmotic stress. These findings support the results of previous research, where increased concentration of exposure is seen to result in more fish mortalities within a population (Andersen et al., 2016). Results of this study indicate increased concentration of exposure significantly increases cell death within gill tissue (**Figure 4.10**), with SDEGs acting in both apoptotic and necroptotic pathways. This dose-dependent action of *P. parvum*, with proposed altered membrane conductance of

effected gill cells with altered exposure concentration (Manning and La Claire, 2010; Moran and Ilani, 1974; Watson, 2001) is in contrast with published literature with regards other phytoplankton. The biotoxicity of *C. marina* in fish through superoxide production from algal cells appears more significantly influenced by fish presence than cell density (Marshall et al., 2003).

The additional SDEG transcription in Moderate and Severely impacted groups, although apparently quite different in specific gene involvement, demonstrate similar overall GO term functionality (**Figure 4.9**). Differential expression analysis between the Severe and Moderate groups identified few genes, suggesting that the transcriptome response within these groups is actually very similar, with similar gene involvement but perhaps a variable magnitude of response.

Regarding GO analysis, the majority of molecular functions and biological processes are shared across clinical presentation groups. Increased severity of clinical presentation therefore does not seem to be accompanied by much additional functionality of gill tissue, rather, it induces a greater number of SDEGs within already enriched responses. Severe group fish demonstrate increased magnitude of response in transcripts associated with cellular stress factors, osmotic control, response to hypoxia and signalling in cell death. This suggests that a greater hypoxic and osmotic challenge is experienced in this group, potentially due to increased toxic insult causing more cell damage and death. Processes such as *antioxidant* and *electron carrier activity* appear of more importance in high concentration exposed groups, with significant GO enrichment of these broad molecular functions within the Severe presentation group (**Figure 4.8**), and significant enrichment of *detoxification* in both Severe and Moderate groups. An increased required for these functions is likely due to greater *P. parvum* toxin exposure.

4.5.7 Divergent clinical presentation in identically treated fish

Previous research has demonstrated the varied susceptibility of rainbow trout to many harmful organisms (Baerwald et al., 2008; Brown et al., 2019; Langevin et al., 2012; Nieto et al., 1984), with tolerance influenced by factors such as age and genetic

providence of fish (Anacleto et al., 2019; Ryce et al., 2005). Varied clinical presentation within high concentration treatment groups in this challenge trial suggested the potential for susceptible and tolerant individuals too in response to *P. parvum* exposure. For this reason, the transcriptome response of fish was examined by clinical presentation rather than exposure concentration, to prevent pooling of samples homogenising any variation in genetic expression between Moderate and Severe groups. Little comment can be made on the influence of the genetic background of fish in this study, although they were outbred stock obtained from an identical farmed source. Regardless, in the end, no evidence was obtained from the transcriptomic results of these fish to support the theory of a susceptible or resistant sub-group of the treated population. With this overall shared GO processes and functionality identified SDEGs in mind, it appears fish within the Severe group perform a similar but amplified pattern of expression to *P. parvum* exposure, rather than any truly inappropriate or failed response. Rather, it appears as though the Severe group represents a population subset experiencing greater *P. parvum* insult.

Variation in toxic insult from *P. parvum* might be due to a number of factors, none of which can be confirmed retroactively. Poor water mixing, concurrent pathology, inactivation of P. *parvum* toxins by bacterial isolates such as *Bacillus subtilis* and *Proteus vulgaris* (Shilo and Aschner, 1953) (either in the environment or gill microbiome) or light-induced toxin degradation (Dafni et al., 1972) might all have played a role in uneven damage to fish. Of consideration too is the potential for varied presentations in the Moderate and Severe groups due to an alteration from acute to chronic stress response. Acute and chronic stress are dealt with by varied physiological and gene expression mechanisms in rainbow trout (Gilchriest et al., 2000; Moltesen et al., 2016). Following an initial exhausted acute response, chronic expression can subsequently dominate in gill expression (Miller et al., 2007). This is due to the varied requirements and functionality of an acute, chronic or exhausted stress response (Balasch and Tort, 2019)The Mild clinically presenting group demonstrate the least impact of exposure, with a transcriptome response most closely associated with that of untreated fish. The Moderate clinical presentation groups appears to represent an intermediary transcriptome profile (**Figure 4.4**), largely similar to the core response

demonstrated by the Mild group with increased gene involvement. The genes identified by differential analysis between Moderate and Severe groups (**Appendix B.1**) suggest a similarity of transcriptomic change between these groups too, with observed differences driven by magnitude of change rather than altered function. The small number of genes (10) that do significantly vary between these groups have function in the inflammatory response, and cell fate. No significant enrichment of biological function could be determined by GO and KEGG analysis for these genes, they do represent potentially useful markers of Severe or Moderate response and might inform future studies interested in a genetic basis of *P. parvum* toxin tolerance in fish. *Gap junction protein alpha 3, LIM Domain 7* and *ras-related protein ral-B* all demonstrate significantly greater expression within the Moderate group relative to the Severe. The products of these genes have functions in maintenance of tissue integrity, cell proliferation and survival, and might represent useful markers in prognostic outcome. Whilst this variation in response might have a genetic basis, considered more likely is a varied severity of insult.

Overall, these results collectively demonstrate that gene expression within rainbow trout gills in response to *P. parvum* exposure is altered by increased concentration of treatment, but the divergence in clinical presentation in high concentration treatment groups of this study is not accompanied by an overall divergence of function. The varied clinical presentation in this study is attributed to spectrum of severity of tissue impact. Greater magnitude of expressional change within the Severe group was observed for genes with actions in hypoxic response, tissue damage and apoptotic functions, supporting the conclusion that fish within this group suffered a greater toxic insult. The varied patterns of gene expression between these groups might then reflect a more acute or chronic response, with more rapid exhaustion of initial response mechanisms (Kienzler et al., 2017).

4.5.8 Applications and future research

Variable severity of clinical presentation observed in this study demonstrates that toxin exposure can elicit both severe and relatively mild clinical symptoms, of which mild is

obviously preferable. Whilst phytoplankton are difficult to exclude from the aquaculture environment, and much research is still needed on the conditions during which blooms propagate to avoid exposure entirely, the identification of ROS as having influence on the toxic impact of *P. parvum* presents an avenue of exploration in amelioration of HAB effect. Ecotoxicology represents an important tool in environmental monitoring and for determining the mechanistic impacts of specific toxins on fish health. However, an improved study design would have incorporated an assessment of gill histology following exposure into the analysis of *P. parvum* impact, so enhanced knowledge of gill damage and confirmation that fish within the Severe experienced greater insult.

Although transcriptomics is highly informative, fold change of genes is unfortunately not always proportionally correlated with translation and downstream protein expression, due to a multitude of factors. Further work might then focus on observing the products of oxidative damage within gill tissue, or proteins with action in detoxifying tissue following exposure. Group genetic drivers of divergence of clinical presentation would also be highly useful, for exploration of the potential for toxin resistant fish, useful in breeding strategies of aquaculture stocks. Different genetic lineages of trout have been demonstrated to be tolerant or intolerant to various toxins in the past. Induction and downregulation of different *CYP450* enzymes and *ABC* genes for variable processing of *P. parvum* toxin, or apparent downregulation of *mucin* activity in severely affected fish might make interesting candidates for further study. These mechanisms might then be exploited in the future, either through selective breeding, or treatment therapies.

Clay treatments have been used previously to treat phytoplankton blooms (Brownlee, 2005) by means of flocculation (Yu et al., 2017) with variable success. Based on results of this chapter, clay (a soil type with generally high cation exchange capacity (Zevenbergen et al., 1997) may confer an additional benefit in inactivation of ROS. As gill damage appears to initiate hypoxic compensatory mechanisms even in mildly impacted fish, ensuring oxygen saturation of the environment of fish should also be considered a priority, even in low density blooms. Sunlight appears useful in reduction in bloom toxicity, so manipulation of light or available nutrients for phytoplankton

might also be utilised in mitigation responses during exposure of fish to *P. parvum* cells (James et al., 2011).

Further work in this subject should include challenge of genetically diverse rainbow trout groups with toxin producing *P. parvum* to determine if a true tolerance might be demonstrated by genetically protected individuals. Additionally, as more information becomes available about the individual toxic components and the molecular structure of toxic proteins produced by *P. parvum*, more might be learnt regarding the action of individual components in initiation of gill response through further transcriptomic studies of elective exposure trials with single toxic moieties. Finally, further work in exploration of this existing dataset might yet yield insight into the action of *P. parvum*, and the gill response to its toxicity.

4.6 Conclusions

The transcriptome of salmonid gills has been extensively studied with regards the response to infectious disease and environmental toxins, however this represents the first study of gene expression in gills following fish exposure to phytoplankton and their biotoxins. Results of analysis suggest rainbow trout suffer deleterious effects such as DNA-damage and oxidation of proteins and lipids following exposure to the toxic products of *P. parvum*, through expression of genes associated with antioxidant response and detoxification. Response to ROS is apparent in tissue, suggesting production of ROS is also an important part of *P. parvum* toxicity. Activation too of innate immune functions within gills indicates an inflammatory response to *P. parvum* and its toxic molecules. Overall, mechanisms of gill response to these toxic products in rainbow trout appear similar to studies in bivalves exposed other HAB phytoplankton. This profile of expression shares similarities with response to infectious disease, as well as chemical stress, but is identical to neither. Although not identified in this study, these results then suggest the potential for future identification of genetic markers of phytoplankton toxic effect in gill tissue.

The available literature on cell death suggests oxidative stress elicits an apoptotic response only whilst cells maintain their reducing capacity against ROS. Excessive ROS accumulation however disrupts homeostasis and induces necrotic-type death instead (Proskuryakov et al., 2003). The transcriptome response altered expression of Nitrous oxide and glutathione transferases as well as pathways of necroptosis activation in fish gills suggest that in addition to apoptosis following *P. parvum* exposure, increased concentration of treatment induces necroptosis. Necroptosis is suspected to be linked to ROS-induced damage, however without challenge of gills to specific toxin components in isolation, this cannot be confirmed. Unfortunately, differentiation of the toxic components of *P. parvum* has not yet been achieved by the scientific community. Regardless however, this chapter presents results as a first step in determining the biological consequences of *P. parvum* toxicity in a nature exposure situation, applicable to both farmed and wild fish

Overall, results suggest that a universal response to *P. parvum* exposure occurs in all fish, with increased concentration of exposure inducing additional involvement of SDEGs, particularly in signalling of cell death. Despite the variation in severity of clinical presentation in high concentration treatment group, no clear difference in the biological processes of these fish was identified. Increased severity of clinical presentation observed likely occurred then due to greater tissue damage by *P. parvum*. The variation in transcriptome response between identically treated Moderate and Severe groups is though still of interest for future research. Variation in expression of *mucin 5* and *mucin 2* is particularly topical, due to their association with other gill diseases, and might represent a target for any future study in tolerance of rainbow trout to *P. parvum*. Overall, these results enhance our knowledge of the interaction of toxin production phytoplankton and gill-breathing fish, specifically aquaculture raised rainbow trout, and highlight interesting avenues of future work.

Chapter 5

5. Variation in the Atlantic salmon microbiome with diverse gill pathology and the influence of on-farm treatments in dysbiosis

5.1 Summary

The adherent microbial communities of gills play an important function in fish defence and homeostasis. Knowledge regarding the components of this microbiota is therefore important for understanding the potential impact on gills following gill trauma and disease. One might expect changes to the microbiome composition of gills in these conditions, particularly if the immune defence of gills is compromised. Recognizing biologically significant changes requires an understanding of what the microbiome in healthy fish looks like, and how it is altered during the lifecycle of fish.

There is limited research regarding the microbial communities of salmonid gills specifically, however what research there is does not provide a consensus regarding the dominant microbial populations of gill tissue. The existing literature suggests that rather than one true microbiome for all 'healthy' fish, microbial populations are labile and vary with environmental conditions, modulated with host need. It is understood though that failure of effective modulation or loss of important components can result in the dominance of less desirable microbes, known as dysbiosis.

This work was therefore performed with the aim of identifying the bacterial populations present on the surface of farmed Atlantic salmon gills out-with a laboratory setting, during the 12-month period following introduction to seawater in aquaculture. Next generation sequencing of the 16S region of microbial DNA was used to assess resident microbial communities of gill tissue. Of interest were differences in community composition between clinically healthy and those with gill pathology, as well as other factors that influenced the remodelling of microbial communities in the studied population. Farmed fish commonly suffer from mixed gill pathologies that impair both productivity and survival, some of which appear to have a microbial component. The association of overall gill health status and microbiome composition in Atlantic salmon

might therefore be important in determining the onset or outcome of these pathologies. There exists too information regarding the specific bacterial species that benefit from and proliferate in instances of impaired fish health, such as occurs in gill disease of salmonids, or environmentally acquired components of the microbiota that might protect from pathology. Therefore, it was considered important to assess alterations to the adherent microbial community of gills in different disease states at multiple taxonomic levels.

Results demonstrate varied microbial community composition throughout the production cycle of studied Atlantic salmon, with significant variation between sampling groups, along with high individual variation. Different levels of gill pathology (determined by histological analysis) appear partially responsible for this variation, through altered abundance of a small number of key microbes. Exposure of fish to hydrogen peroxide was identified clearly too as a factor in the restructuring of the gill microbiota between sample groups during the production cycle of the study population. Broadly, this study enhances the available scientific information regarding the composition of the gill adherent microbial communities of Atlantic salmon in a farmed situation during marine production. Overall, results demonstrate that community composition during the production cycle is influenced by a number of variables, including gill pathology, and that the consequences of these variables must be considered when deciding on therapeutic or production-associated treatments in salmonids, as the balance of the microbiome appears easily disrupted, and may have important consequences for fish health.

5.2 Introduction

5.2.1 The microbiome of fish

Mucosal surfaces of fish are important not only in their physiological function, but also as essential barriers against infection. Gills specifically form a physical barrier, with production of mucus further facilitating their function and immunological defence against the external environment and pathogenic organisms (Esteban, 2012; Peatman et al., 2015). All mucosal surfaces host communities of indigenous microbiota known as the microbiome, predominantly bacterial organisms that survive within the unique environment of the mucosa (Gajardo et al., 2016; Llewellyn et al., 2016; Merrifield and Rodiles, 2015). These microbial communities have been suggested by researchers as important in a number of host functions, and appear to be crucial for the continued health and survival of their hosts (Dominguez-Bello et al., 2019; Mohajeri et al., 2018; B. Wang et al., 2017). Mucus contains defensins, immunoglobulins, lectin-like agglutinins, lysozyme and a variety of additional peptides with antimicrobial activity that protect fish and maintain microbial homeostasis (Esteban, 2012).

The microbiome is suggested to act as the interface between a host and its environment, with the immune system restricting microbiota to their niches (Beck et al., 2015). The gills are a mucosal surface in constant contact with the environment and the microbes present therein, with the hosted community of resident microbes apparently influenced by both the immune function of their host, as well as microbial interactions and environmental factors (Egerton et al., 2018; Lokesh and Kiron, 2015; Ornelas-García et al., 2018; Reverter et al., 2017; Webster et al., 2018). Much of the existing research regarding the microbiome of fish is focused on the activity of GIT microbiota, particularly in salmonids. This research suggests that microbial communities are specialized between species and that an unperturbed microbial community of the gut may assist in functions like digestion, immune defence and even neurological signalling for the continued survival of their host (Foster et al., 2017; Roeselers et al., 2011; Zha et al., 2018). Microbes in turn obtain benefit from their host in the form of a protected, nutrient-rich environment (Zilber-Rosenberg and Rosenberg, 2008). Modulation of the

microbial community occurs with a multitude of environmental variables and stressors, including different diets and aquatic environments. These alterations can be considered as positive adaptation to change, but it appears that negative change can also occur.

5.2.1.1 Microbial communities of salmonid gills

The majority of previous research in microbial communities of fish has focused on intestinal populations, due in part to the suggested importance of these microbes in the productivity of fish. There does exist, however, a body of research regarding the microbial populations of external epithelial tissue, including skin and gills, and a growing focus on the importance of these bacteria, as gill disease continues to severely impact aquaculture of a variety of fish species. Early culture-based research suggested that the main microbial communities of fish were similar to the surrounding water (Al-Harbi and Uddin, 2005; Mudarris and Austin, 1988; Nieto et al., 1984), but more recent DNA sequencing-based studies have determined that the adherent microbial communities of salmonid gills are specialized and distinct from environmental populations (Legrand et al., 2018; Pratte et al., 2018). Results vary with regards to community composition in different species of fish, and with different environments. The microbial communities of gills appear more similar to surrounding environmental water populations than those of the gastrointestinal tract (Nedoluha and Westhoff, 1997), closely resembling that of the skin epithelial communities (Wang et al., 2010), although still distinct. Key differences in community composition between skin and gills (Legrand et al., 2018) may be due to a varied function.

As with bacteria of the GIT, community composition of gills can be altered by environmental variables (Masouleh et al., 2006), and seasonal trends are described (Al-Harbi and Uddin, 2008). Gill tissue appears to host a number of bacteria with antimicrobial properties, hypothesised to act as part of the defence of gill tissue to harmful bacterial isolates (Kanno et al., 1989; Ringø and Holzapfel, 2000). A role for gill bacteria in removal of ammonia is also proposed (Van Kessel et al., 2016). Much though is still to be learnt regarding a functional role and potential benefits to the host of microbes present within the microbial communities of gills.

A function of particular interest in the aquaculture production of salmonids then is the ability of microbiota to assist in the immune and stress response of their host to negative stimuli, such as disease. Commensal microbes within adherent populations are known to compete with others for resources, and even produce compounds such as anti-microbial peptides that impair the growth of microbial competitors (Gomez et al., 2013b; Wanka et al., 2018). This might act in the interest too of their host, through preventing of overgrowth of any single bacterial population, and inhibition of the activity of bacterial pathogens (Kamada et al., 2013). Components of the microbiota might further assist in the host response to stress through production of protective compounds during xenobiotic exposure that limit damage to host tissue (Carmody and Turnbaugh, 2014). Microbes are even suggested to confer direct pathogen resistance to their hosts through specific priming of the host immune system other species (Montalvo-Katz et al., 2013). The loss then of this function by disruption of the microbial community might have serious consequences of the health of fish.

5.2.2 Dysbiosis: disruption of resident microbial communities

Interaction of the microbiota can be beneficial to the host, but it can also have negative consequences. The high plasticity of resident microbiota allowed adaption to changing conditions, but change can also be disadvantageous. Dysbiosis refers to the maladaptation or microbial imbalance of resident microbial communities, defined as unfavourable alteration resulting in or as a consequence of disease (Petersen and Round, 2014). Dysbiosis is therefore associated with negative consequences for the host (Reid et al., 2017). Alterations to microbial structure is a documented consequence of disease in many organisms (Brugman et al., 2018; Crakes and Jiang, 2019; Gram and Ringø, 2005), and is considered a risk factor for impaired welfare and survival of fish (Reid et al., 2017).

Disarrangement of the healthy microbiota can result in loss of symbionts, with resulting altered homeostasis and loss of immunological function, allowing overgrowth of less desirable microbes. Bacteria known to be harmful have been demonstrated to proliferate

alongside loss of commensal host microbiota in dysbiosis, often to the apparent detriment of microbiome function and with clear consequences for host survival (Honda and Littman, 2012). Microbes considered opportunistic pathogens and even directly infective bacteria known as pathobionts can be present in low abundance as part of the apparently healthy microbiota, proposed to even assist with signalling and other functionality at the mucosal surface (Chow and Mazmanian, 2011). Altered abundance of these bacteria however can lead to a negative impact on fish health.

It is though difficult to differentiate any advantageous adaptation from dysbiosis within the highly plastic microbiome. Some indicators of dysbiosis though might include predominance of genera associated with infectious disease (Llewellyn et al., 2017), or a negative biological outcome for the host. Health status of the host is often utilised to differentiate groups, although this does come with the associated problem of the chicken and the egg; does disease beget dysbiosis, or dysbiosis beget disease?

5.2.2.1 Dysbiosis and disease

Microbial change in salmonids has been reported in response to viral and parasitic infection, as well as with bacterial disease (Llewellyn et al., 2017; Reid et al., 2017; Toranzo et al., 1993). The microbiota-host immune system interaction is considered highly important in determining the outcome of these infections, due at least in part to the proposed association of microbes in mucosal defence (Honda and Littman, 2012). The impairment of the immune response that occurs with stress and disease therefore appears a key factor in alterations to community composition (Uren Webster et al., 2018).

Despite the extensive research into the microbiome of many host species, and increasing knowledge regarding dominant populations, the function of the taxa within the microbiota is still unclear. Disarrangement of microbial communities in dysbiosis appears to involve out-competition of 'normal' benign or possibly beneficial taxa by those less favourable to host survival. Proliferation of microbes such as clostridial bacteria that produce toxins within the GIT is proposed to elicit gut motility and aid

digestion, but they can also cause harm through neuronal damage (Yang and Chiu, 2017), and appear to flourish in situations of GIT dysbiosis (Shaw et al., 2019). Obligate symbionts are proposed to contribute to the overall function, including immunity. Research shows that resident microbiota can also function directly in prevention of overgrowth of pathogen such as Tenacibaculum in fish (Wanka et al., 2018). Increased pathogen susceptibility through antibiotic clearance of 'normal' microbes is then a concern too in fish, with treatment demonstrated to increase the risk of various bacterial infections (He et al., 2017).

Perturbation of the functional community structure in dysbiosis can also have predictive values in disease. Alongside studies of genetic biomarkers of altered host immune function, a number of studies concerning the human microbiota have identified microbial biomarkers of disease (Segata et al., 2011). Microbes can be utilised as indicators of disease recovery or recurrence (Sobel et al., 2019), as well as prognostic indicators in disorders involving human cell malignancy (Meng et al., 2018). Some specific examples include *Streptococcal peptidoglycan*, a proposed antigen of the altered immune state of psoriasis in humans, that is considered a factor in onset of this inflammatory skin disease (Baker et al., 2006). Presence of Clostridiales and *Bifidobacterium* are considered biomarkers of recovery from necrotizing enterocolitis in preterm infants, associated with improved intestinal barrier function (Ma et al., 2018). Lactobacillus relative composition was determined to be a prognostic indicator in recurrence of bacterial vaginosis (Sobel et al., 2019), and Crohn's disease is thought to have a distinct microbial signature (Pascal et al., 2017). The list goes on.

The field of disease-associated dysbiosis research has rarely been extended to the study of fish, however it has the potential to greatly inform teleost research, particularly in aquaculture-reared salmonids. Farmed fish suffer frequently from gill pathologies of complex aetiology, and the alterations with disease and potential involvement of adherent microbiota is unknown. Highly economically important diseases such as AGD have been suggested to have as yet unestablished microbial involvement (Bowman et al., 2004; Embar-Gopinath, 2006) that merits further exploration. The factors associated with varied pathogenicity of apparently identical organisms in different fish might also

involve a microbial component in their predisposition. Previous research stresses the need to establish a causal link between imbalances in the microbiome and pathology in teleost fish (Llewellyn et al., 2014).

5.2.2.2 Prevention of dysbiosis

The microbiota of salmonids is both complex and dynamic, with an apparently important role in overall functionality of the mucoid layer (Sar and Rosenberg, 1987). Recent studies have investigated native microbiota for their potential in disease treatment (probiotics) or disease prevention (prebiotics) in the GIT of fish, based on the success in human research (Carnevali et al., 2016). Probiotics in the guts of teleosts are often lactic acid bacteria for digestion, including *Bacillus, Lactococcus, Shewanella,* and *Aeromonas* bacteria (Burr et al., 2005; Merrifield and Carnevali, 2014), however manipulation of immune function through probiotics has also been reported (Carnevali et al., 2016; Cordero et al., 2015). Immune modulatory probiotics in the GIT include *Pediococcus, Bacillus, Clostridium* and *Shewanella* genera against *Vibrio* and viral infections (R. Ferguson et al., 2010; Huang et al., 2014; Sakai et al., 1995). Endogenous microbes have been proposed to interfere with pathogen colonisation (Ringø and Olsen, 1999), demonstrated as interfering with colonisation by *Flavobacterium* (Boutin et al., 2012), *Aeromonas* and *Enterobacteria* (Araújo et al., 2015; Didinen et al., 2018; Ringù et al., 2000), infectious microbes capable of causing serious tissue damage.

Studies investigating the epithelial-associated communities of Atlantic salmon have obtained varied results regarding the dominant bacterial populations, consistent with previous hypotheses and observations that microbial communities are modulated with environment, and individual host requirements. Indeed, a high degree of individual variation is possible between individuals within a population, with gene expression demonstrated as a factor in modulation of adherent bacteria (Boutin et al., 2013b, 2013a). While obligate symbionts are proposed as essential for host survival, facultative microbes are those considered to facilitate more nuanced adaptation. Dysbiosis is often accompanied by an overall reduction in microbial diversity (Petersen and Round, 2014),

an observation that might represent the loss of both symbionts and facultative bacteria. Varied microbial populations with different disease states might represent either dis-advantageous, pathological change, or advantageous change for enhanced survival and transfer of ecologically important traits, depending on the activity of the microbes. A multi-layered microbiota, with core and more flexible, transient microbial populations, might then be key to host fitness. An understanding of the specific taxa altered with exposure to stressors might therefore be crucial in differentiating adaptive change from dysbiosis.

5.2.2.3 Factors influencing dysbiosis

The microbiome drives adaptation to environmental factors, or even disease challenge. The methodology of production in aquaculture, however, likely limits the function of this adaptation, through selection of fish for desirable production traits and use of genetically limited broodstock, a concern in all salmonid aquaculture (Burton et al., 1980; Cross and King, 1983; Withler, 1988). Hatchery-reared salmon have been demonstrated to lack the core microbiota of wild counterparts, with lower microbial diversity for divergent community structure and function (Webster et al., 2018). The bacteria as part of the transient, horizontally acquired members of the microbiota seem likely candidates as factors in prevention or exacerbation of disease such as gill pathologies in the farmed fish situation, but the production environment and genetics of hatchery fish might prevent the acquisition of these microbes. This has serious implications for the fitness of hatchery-reared fish, potentially predisposing them to dysbiosis and enhanced likelihood of infective disease.

In addition to their divergent microbial community composition, aquaculture presents a unique set of challenges to fish health.. Stocking density and stress are known to impact microbial community composition (Brown et al., 2019; Legrand et al., 2018), as is the application of antibiotics (Higuera-Llantén et al., 2018). Traumatic damage to tissue or any variable that impairs gill function will likely perturb the immune function of gills, and so the modulation of resident microbiota. The impact of other on-farm activities on

the gill microbiome remains to be explored but might present crucial information regarding the apparent susceptibility of farmed fish to complex disease pathologies.

Freshwater and marine environments host markedly different populations of microbes (Logares et al., 2009; Methe et al., 1998), and so exert different infectious challenges on fish. This might be of particular concern during transition from fresh to salt water for salmonids such as Atlantic and Pacific salmon, a transition considered highly stressful (Price and Schreck, 2003) and following which high mortality and disease incidence is experienced by farmed salmon (Balseiro et al., 2018) . Microbial alterations to surface epithelial communities and the GIT have been observed in salmonids during this transition (Dehler et al., 2017b; Lokesh and Kirin, 2016). These altered mucosal microbiomes likely represent adaptation to an altered environment with incorporation of surrounding microbes, but it might also represent negative change with stress induced impaired immunity, with opportunistic colonisation and resulting dysbiosis.

Fish farmed in the marine environment, such as aquaculture reared Atlantic salmon, are exposed to a multitude of environmental factors and infectious agents capable of eliciting gill trauma and disease. Not only do the high stocking densities and intensive production predispose to conditions less commonly encountered in wild counterparts (Assefa and Abunna, 2018), exclusion of pathogens from the marine cage environment is extremely difficult. Gill trauma can result from exposure to harmful organisms such as cnidarian jellyfish (M. D. Powell et al., 2018), harmful phytoplankton (Díaz et al., 2019; Yang and Albright, 1992) or sea lice (Pike et al., 1999). Even aquaculture specific challenges such as handling or high concentrations of suspended solids (Rodger et al., 2010) can cause damage to gill tissue to predispose to infectious disease.

5.2.3 Gill pathology

A number of gill pathologies are considered to have significant negative effect on Atlantic salmon production. In addition to infectious outbreaks of bacterial disease and AGD, generalised gill diseases are frequently reported (Steinum et al., 2010). Fish reared in the marine environment are rarely exposed to just one agent of disease, and so

observed pathologies are often caused by multiple infective agents. Gill diseases in farmed Atlantic salmon are therefore often complex conditions, related to a number of concurrent factors. Whilst some of the pathologies described appear to involve microbial infection, altered microbiota has been demonstrated to occur with many infectious diseases.

Directly infective pathogenic bacteria such as *Tenacibaculum, Vibrio, Aeromonas, Bacillus* and *Pseudomonas* can cause gill disease in salmonids (Belkin et al., 2005; Saad and Atallah, 2014), and so too can opportunistic infections. Microbes known as opportunists are present within the environment or microbiome without any observed association with clinical disease in the majority of circumstances. These microbes are capable though of causing disease in specific circumstances, such as ones that result in stress or impair immunity (Belkin et al., 2005; Price et al., 2017). Concurrent disease that weakens immunity can allow bacterial overgrowth, with associated negative consequences. Gill disease, particularly in farmed salmonids, is often considered a complex infection, with multiple compounding factors influencing whether clinical disease is seen. Even AGD, a gill condition relatively simple to diagnose, appears to be compounded by microbial variables that influence disease outcome (Bowman et al., 2004; Embar-Gopinath, 2006). Occasionally a single infective agent can be blamed for disease, but more often a multitude of potentially damaging organisms or factors appears to result in the gill disease observed. Diagnosis of a single agent of pathology is therefore extremely difficult and at times misleading when investigating disease in marine cage production systems of Atlantic salmon.

5.2.4 Investigating the gill microbiota of farmed Atlantic salmon

This chapter of work sought to investigate the adherent microbiota of Atlantic salmon gill tissue obtained from a marine cage system of production. Fish were commercially reared salmon exposed to a variety of environmental conditions and infectious agents throughout the one-year study period. This is likely representative of the majority of aquaculture reared Atlantic salmon. Of interest were the changes in microbial community composition through the study period, and any association of specific

microbial agents with greater severity of gill pathology. This study sought to identify and characterise any incidences of dysbiosis, with the aim of observing the microbiota associated with healthy and diseased gill tissue. Instead of identification of a specific agent of gill disease, histological sections were appraised and scored based on an existing described scoring system (Mitchell et al., 2012) to provide for each fish a classification of the level of gill pathology. Gill pathology could then be used as an indication of functional compromise, and assumed associated immunocompetency, to characterise fish gills are 'healthy' or 'diseased'. Little information is currently available regarding the microbial communities of Atlantic salmon gill tissue, healthy or otherwise, so this was a topic this research sought to begin to address.

Results demonstrated a clear group effect on samples, with fish obtained at the same timepoint most similar in their microbial community composition. Individual variation in adherent microbiota was however present, and at least partially explainable by the disease state of gill tissue. From this, a number of genus-level trends in community composition with gill disease were identified. Although a number of confounding factors during the production cycle make identification of a specific agent of microbial change problematic, results are representative of fish reared in the marine environment, and the challenges they encounter. Results demonstrate variability of the obtained microbial isolates during the production cycle, supporting the theory of constant challenge and requirement for adaption in gill tissue. A number of identified on-farm events appear to have induced microbial community alterations during the sampling period, with particular focus made on the results of hydrogen peroxide treatment of fish.

5.3 Methods

5.3.1 Sampling

An in-depth description of the sampling regime is provided in Methods (Section 2.2.1). Briefly though, samples were obtained from Atlantic salmon in the marine stage of aquaculture production from a single sea cage at the Loch Spelve SSF marine site. Sampling was conducted over a 12-month period, May 2017 – June 2018, with 12 fish collected during each visit, and a total of 132 fish sampled overall.

Fish were euthanised for collection of material to be used in two chapters of work for this thesis. Collected gills were not washed or handled in any way prior to fixation other than to remove them from fish using sterile technique. Samples utilised in this chapter were gill sections from the left side, first gill, fixed in RNAlater solution (ThermoFisher Scientific) and gill tissue from the left side, second gill fixed in formalin. Data were collected regarding the gross clinical appearance of gill tissue, as well as any other observed external or internal pathology. Fish were individually identified using a numbering system. For example, fish 3F6 was obtained on visit 3, and was the sixth individual sampled.

To explore bacterial community composition in the gills of farmed Atlantic salmon over an annual production cycle and for investigation of potential markers of gill pathology, samples of 12 fish were taken at 11 different time points (n = 132). Gill sections were fixed for both histology and DNA preservation, so DNA could be extracted and partial 16S microbial genomic region sequenced. Additional measurements were taken from fish during sampling, including weight, ‘clinical-level’ lesions observable in gill tissue and any other apparent gross pathology, both internally and externally. Weather conditions and general environmental parameters were noted at each time point. Environmental parameters such as water temperature, pH, oxygen saturation and salinity were monitored constantly by the producer, however this data was not made available in time for analysis and are therefore not included in these results.

DNA extraction yields were within the range of 80 – 200 ng/µl for all utilised samples. Lower weight tissue sections were utilised to minimise protein contamination and PCR inhibition experienced in earlier, pilot extractions. Multiple extractions from individual fish were then pooled to maximise microbial genomic yield. Of the 132 biopsy-derived DNA samples utilised, eight failed to yield adequate PCR products. These were from the first and final sampling groups, with human error considered the likely cause of failure. The sequencing of these samples was repeated, however not in sufficient time for inclusion in analysis.

5.3.2 DNA extraction and sequencing

Sections of gill tissue including cartilage and lamellar tissue were removed from the central gill arch and DNA extractions as described in Methods (Section 2.3.2). Initial extractions from RNAlater fixed tissue contained high levels of protein contamination, with poor performance in pilot study polymerase chain reactions (PCRs), prompting the inclusion of the phenol/chloroform step (Section 2.3.3). Use of the phenol/chloroform step in extraction of DNA greatly enhanced the success of downstream PCR reactions. An initial problem with DNA integrity was resolved by removal of the vortex step from phenol/chloroform extractions due to its suspected action in DNA shearing. Ethanol fixed gill tissue was not utilized due to the higher variability of DNA extraction success from this fixative.

Pooled DNA extractions from fish 9F1, 9F2, 9F3 and 9F4 along with the negative spin control obtained during DNA extraction were prepared and sent for commercial next generation sequencing at ZymoBIOMICS Targeted Sequencing Service (Zymo) with primers targeting the V3/V4 region. Resultant sequences from Zymo were, however, determined to be of high cost and potentially poor quality (with low read count and ASV richness). Further sequencing work was therefore performed in-house, for enhanced control and to gain knowledge of additional laboratory techniques, with the technical assistance of Dr Adam Wyness (AW).

Next generation sequencing of 129 tissue samples, with additional environmental samples and controls was performed using the manufacturer's protocol (Illumina, 2013), modified to maximize output from DNA samples. Trial amplicon PCRs and clean-ups were conducted to optimize these protocols prior to use on all samples. PCR's of variable cycle number were performed to assess the impact of cycle number on template amplification. More cycles result in a greater PCR yield for downstream use, but PCR can also bias the results of microbiome research (Kebschull and Zador, 2015). Following trial AMPure XP bead (Agencourt, Beckmann Coulter) clean-ups in reduced volumes of elution, volume of elution for the first clean-up of the protocol (Section 2.6.2) was reduced as it was determined DNA yield was maintained whilst achieving a greater per volume concentration. Finally, the protocols for index PCR in attachment of sequencing primers and multiplex markers were also modified, with replacement of water in the reaction mixture with an increased volume of template (Section 2.6.3). Full details of all steps in library generation and preparation for next generation sequencing are provided in Methods (Section 2.6).

Sequencing was performed using a Miseq Illumina sequencer. The sequencing mixture contained a 20 % spike-in of pre-prepared PhiX sequencing control. This relatively high level of PhiX spike-in was used to maximise quality of sequences obtained from a predicted diversity library (based on results of Zymo pilot sequencing and assumed high host genomic material contamination).

Firmicutes and Gammaproteobacteria in particular are known to have a high 16S copy count (Větrovský and Baldrian, 2013), a fact that might alter community abundance estimates in this research. PCR can introduce additional bias through magnifying this difference in apparent abundance, making it appear as though these microbes are dominant when they are not. Although this bias was certainly a potential factor in this research, identical treatment and PCR of all samples ensured at least an identical bias, so fluctuations in relative abundance between groups remained meaningful.

A low volume for elution (25 µl) was used in the clean-up step following amplicon PCR of the protocol. By using a low volume, the concentration of DNA obtained could be

maximized, with very little of the final volume required for further steps. Trial clean-ups with this low elution volume were performed on test PCRs to ensure yield was maintained prior to application of this protocol to samples intended for sequencing. The protocol for the next PCR, for attachment of Illumina sequencing adapters and dual indices, was also modified. Illumina recommends an input volume of 5µl of genomic material, with no indication of desired concentration. Previous publications utilizing the 5µl volume have rarely report their DNA yield. Due though to the low yield of microbial DNA from the initial amplicon PCR in this study (mean = 1 ng/µl), presumably due to high host DNA masking, it was decided to maximise the input volume of genomic material in index PCR. Triplicate amplicon products were pooled following clean-up to produce a DNA concentration of the maximum achievable for all samples, 1ng/ µl. The index PCR protocol was then modified so that instead of 5 µl of genomic material and 10 µl of DNA-free H_2O, 15 µl of genomic material was used. Use of biopsy-derived extractions was clearly problematic even at this stage with regards to high host DNA contamination and proportionally low microbial genomic material inclusion. These steps did, however, maximise input concentration of DNA for sequencing(his step in the protocol at least appears not to have introduced additional bias or negatively impacted results in any way).

Finally, for sequencing on the Illumina MiSeq system, a modified 20 % spike-in of pre-prepared PhiX sequencing control was added to the library, as initial pilot results obtained from Zymo suggested a low diversity of bacterial samples. Reads within low diversity libraries are often incorrectly classified as being of low quality due to the lack of variation identified by the machine during sequencing, and inclusion of PhiX adds diversity to the library. A higher PhiX concentration means less obtained sequences, however appraisal of the rarefaction curve in Qiime2 suggests adequate sequencing depth was achieved regardless. In hindsight, results of sequencing demonstrate the diversity is particularly low in the group 9 samples utilised in pilot sequencing, and such a PhiX step might not have been necessary.

5.3.3 Computational analysis and figure construction

Following pre-processing and taxonomic assignation using Qiime2 v2017.2 (Bolyen et al., 2019; Caporaso et al., 2012) and DADA2 (Callahan et al., 2016), ASV results were obtained for the gill dataset in favour of Operational Taxonomic Units (OTUs) (Callahan et al., 2017). Full details in Methods (2.6.5). Taxa were assigned to lowest taxonomic level using both SILVA 128 (Quast et al., 2013) and Greengenes 13_8 (McDonald et al., 2012) reference databases. Databases have been demonstrated previously as imperfect, lacking taxonomic information or incorrectly identifying sequencing results (Lydon and Lipp, 2018). Differences were observed between obtained taxonomic classification in this instance also. SILVA 128 (Quast et al., 2013) was utilised to taxonomically classify isolates for downstream analysis in this study as it appears to more reliably confirm lower taxonomic classification of ASV's. A single ASV of proportionally high abundance in results assigned only higher taxonomic identity was independently investigated and confirmed as bacterium *Procabacteriaceae.* Chloroplast, archaea, mitochondria and results unassigned below kingdom level were removed for generation of the final dataset.

Abundance profiles were calculated based on total read counts in individual samples for assessment of beta diversity. Beta diversity metrics and multivariate analysis were performed using the programs Primer version 7 (Clarke and Gorley, 2015) and Permanova+ (Anderson et al., 2015). Additional figure generation and statistical testing was performed using Vegan and Bioconductor packages in R 3.5.0 (R Core Team, 2018). Alpha-diversity metrics were calculated for treatment medians of a rarefied dataset obtained through Qiime v2017.2 (Bolyen et al., 2019; Caporaso et al., 2012). Resemblance matrices were obtained through use of the analysis software program Primer version 7 (Clarke and Gorley, 2015) for Bray-Curtis similarity analysis between samples. Matrices were used in various downstream analysis including principal coordinate analysis (PCO), hierarchical clustering and ANOSIM, alongside square root transformed abundance data for PCA. Permanova + was utilised for statistical testing. Figures were generated using Primer version 7 and R. Alpha diversity indices (richness, evenness, Shannon's diversity index and Simpson's diversity index) were obtained

based on both rarefied and non-rarefied datasets. Rarefaction was performed to a sequencing depth of 1200, and diversity indices were subsequently calculated using Vegan package in R and the inbuilt functions of Primer 7. Statistical analysis of data separated by group (groups 1 to 11) and histological score assessment (None, Mild, Moderate or Severe / Healthy, Diseased) at various taxonomic depths were performed using innate R functions for t-testing and anova.

5.3.4 Histology

Histological, H&E stained sections were obtained from formalin fixed tissue for microscopic assessment. Assessment was performed using a light microscope first at low magnification for general overview of tissue, and then at high magnification to determine the details and extent of any tissue pathology. The scoring system used (Mitchell et al., 2012) provided a framework for classifying tissue of fish gills as having 'none', 'mild', 'moderate', or 'severe' pathology. Only scoring by the author is included in this thesis. Scores were generated based on 24 gill-pathology features (overall score and select infectious indices provided in **Appendix C.1**).

5.4 Results

5.4.1 Gross pathology and clinical observations

Fish were obtained consistently from the same population, from a single net pen within the specified farm location. As such, fish increased in age and weight with each sampling, beginning at an average weight of 84.8g and finally reaching an average weight of 3463g (**Appendix C.2**). Due to the variable age and weight of fish, a body condition factor was not calculated, however notation was made of fish considered 'runts' by the producer's standards (i.e. in particularly poor condition, with high length to body width ratios). A total of 10 fish considered 'runts' were sampled during the 12-month trial, fairly evenly distributed across the majority of sample groups. These fish were not excluded from the sample population as it was considered that these 'runts' are still representative of the population as a whole. Additionally, those with poor body condition might be those most susceptible to gill pathology, and therefore of particular interest in this study.

Assessment of gross pathology of gills was performed, with visual appraisal of all gill arches on both sides. Observed disease was described as either AGD, general gill pathology or a combination of both. General gill pathology refers to gill lesions of unknown aetiology, including but not limited to lamellar shortening, petechia and discolouration. Lesions described as AGD-associated were disease-typical mucoid plaques on the surface of gills. The majority of gills presented with a mixture of both, with similar pathologies that were observed to worsen seasonally, as previously described (Downes et al., 2018; Gunnarsson et al., 2017). Illustration of frequently observed gill pathology is provided alongside total data collected for gross pathology during the study duration (**Figure 5.1, 5.2)**. Additional clinical assessment was also performed, with documentation of any external and internal pathology. Rare incidences of internal pathology were occasionally observed throughout the trial, including liver paleness and mild peritonitis, as well as melanosis and adhesions typically associated with vaccine injury. Externally, unilateral and bilateral cataracts were observed,

particularly in younger fish, with these apparently resolving with time. Sea lice were also observed and documented, detailed here only as presence/absence. (**Appendix C.1**).

Gross pathology was identifiable in gills from the beginning of the trial and initiation of sampling (**Figure 5.1).** AGD-associated lesions were first identified in the second sampling group, in June, with an increasing proportion of affected individuals and increased severity of infection through to September. Number of impacted individuals and severity of disease then reduced during winter, with AGD-lesions then again beginning to worsen from March onwards, consistent with previous data on the global seasonality of AGD as a marine infection (Rozas et al., 2012; Wright et al., 2018). General gill pathology was identified in fish at the point of their introduction to sea water, suggesting pre-existing pathology from the freshwater environment. Gills were again more severely impacted in September and November following warm summer temperatures, with few fish identified as entirely without gross pathology through the sampling period.

Figure 5.1 Gross lesions in gills

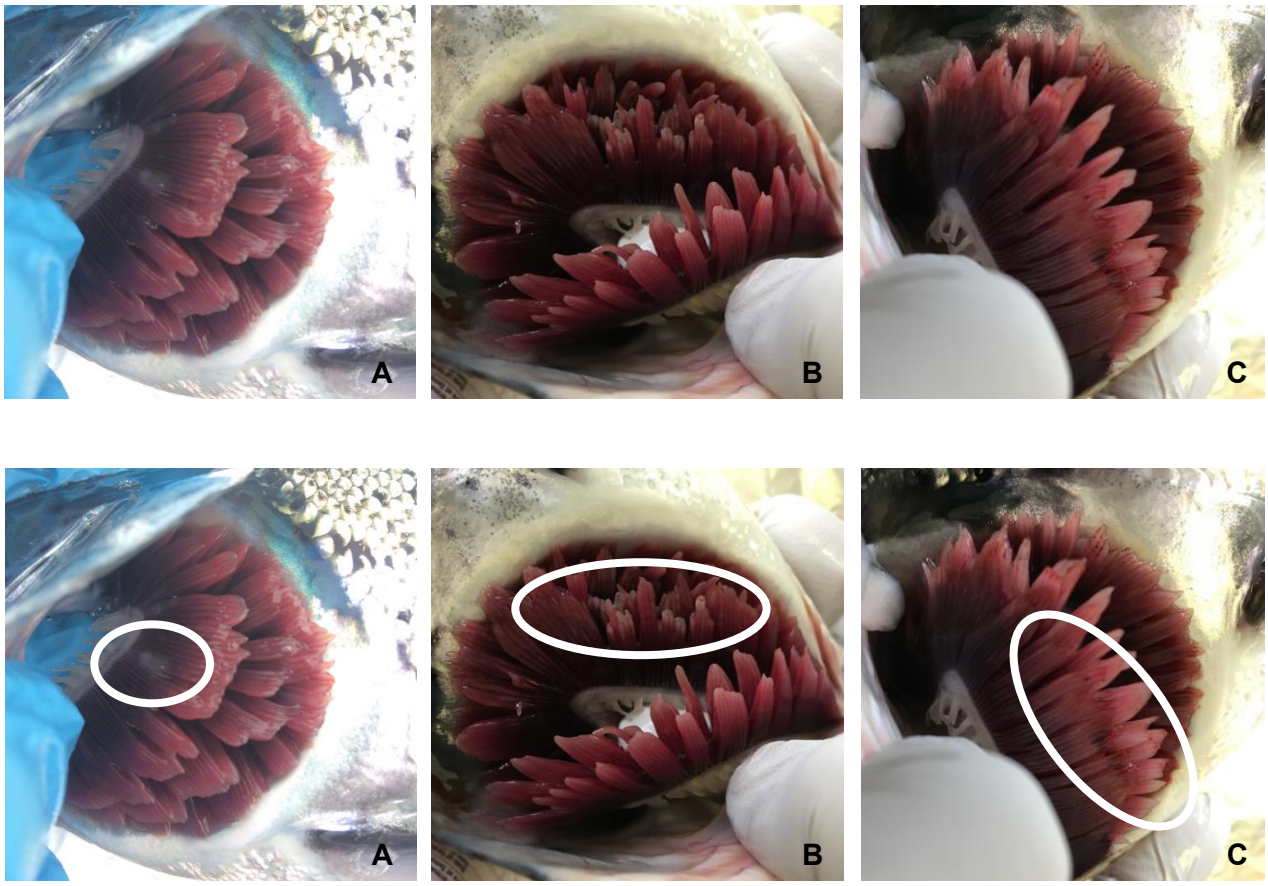


Figure 5.1: *Gross gill pathology observed during sample collection. Examples of localized AGD (score 2, image A), multifocal generalized gill pathology (score 3, imagine B) and localized generalized gill pathology (score 2, image C). Gills in image A demonstrate stereotypical mucoid plaques associated with AGD on the first gill only. Gills in image B demonstrate lamellar shortening and discolouration indicative of a number of gill pathologies. Gills in image C demonstrate petechial haemorrhages within the gill tissue localized to distal lamellar tissue. General gill pathology and AGD were classified in gill tissue using a numerical scoring system (0 – 4; none to extensive pathology).*

Figure 5.2 Trends in gross pathology

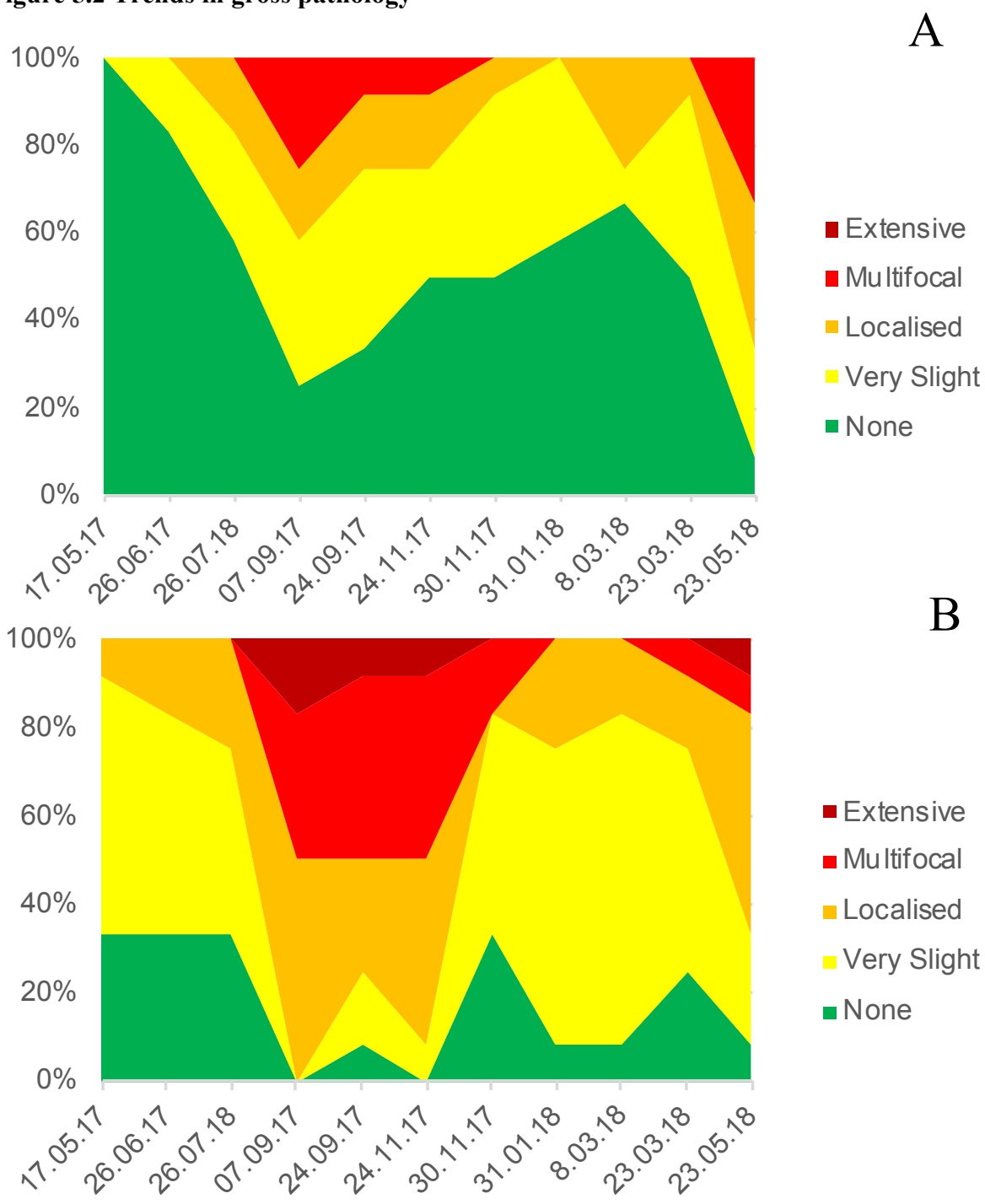


Figure 5.2: *Figures illustrating the distribution of scores for ADG (A) and generalised pathology (B) within samples. Seasonality of severity is observable across gross lesions, with onset of AGD following fish entry to sea water, but presence of other pre-existing pathology in gills prior to entry*

5.4.2 Histopathology

Histological assessment of all sampled fish was performed using the semi-quantitative scoring system previously described (Mitchell et al., 2012). A numerical score was thus achieved for each histological section, so observable histopathology for each fish could be characterised as 'none', 'mild', 'moderate' or 'severe'. Trends are observable throughout the 12-month dataset, with scores rising from the initiation of the sampling period to most severe pathology being observed in sampling visits 4 and 5 (between September and November 2017). Scores then gradually decreased; however, they do not reach the same low levels observed at the initial introduction to saltwater (**Figure 5.3**). Exemplar images of gill sections demonstrate the classifications used to categorise fish in this study (**Figure 5.4**). Disease-specific lesions are highlighted in each image, alongside general pathology that contributes to the numerical scores obtained.

Beyond apparent seasonal trends in altered gill health, variation in severity of observed pathology was identified within sampling groups. Fish within a number of sampling groups diverge in their histological score from the average group score, indicating the presence of variable levels of gill disease within the sampled population. Overall, gill sections were observed to contain many of the characteristics utilised in the selected scoring system. A number of histologically observable pathologies were identified though that were not accounted for in the gill scoring system. Although detailed histological appraisal was made of each tissue section, the details of these additional pathologies could not be included in the final numerical scores.

Figure 5.3 Seasonal variation in histology

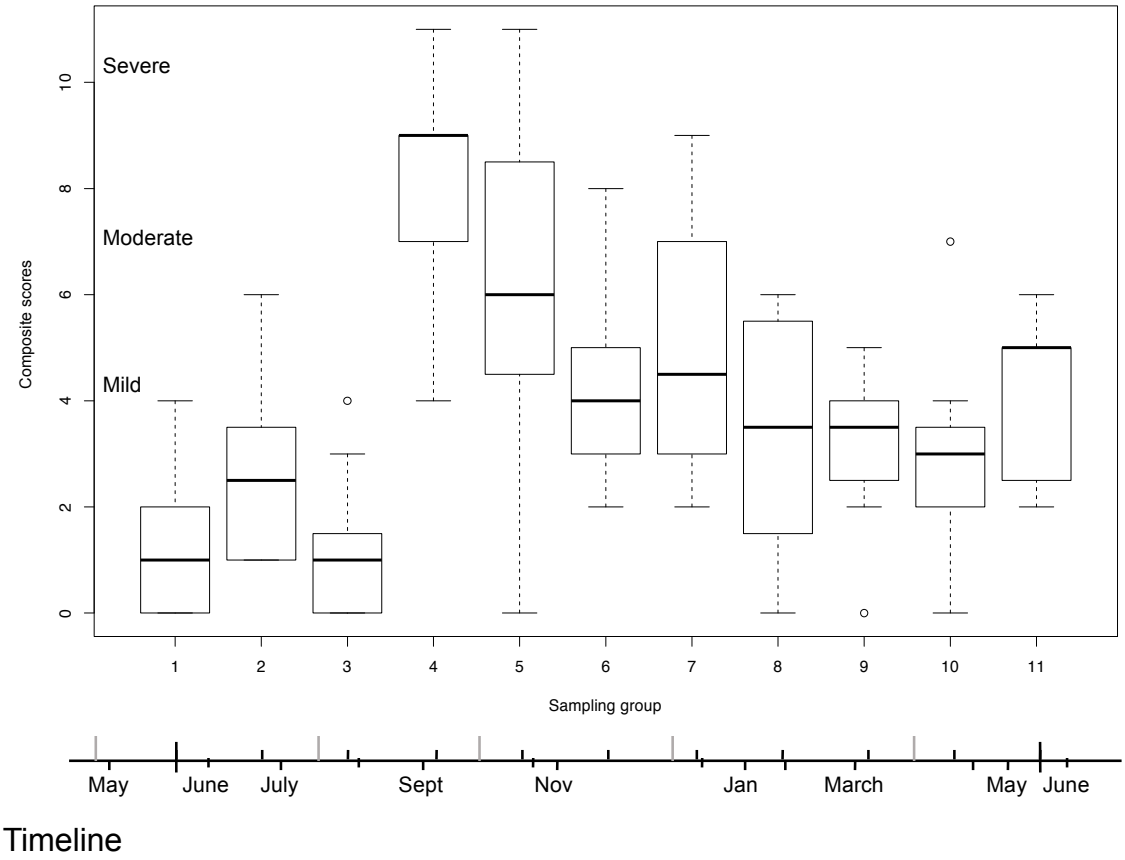


Figure 5.3: *Gill histology scores obtained from fish collected over 12-month sampling period. Composite numerical scores are illustrated for each sample visit along the y axis, and visit number is detailed by the x axis. Sampling timeline illustrates at what point various on-farm events such as hydrogen peroxide (H_2O_2) treatments were applied to fish, alongside timing of sample collection.*

5.4 Figure 5.4: Histopathological change classifications

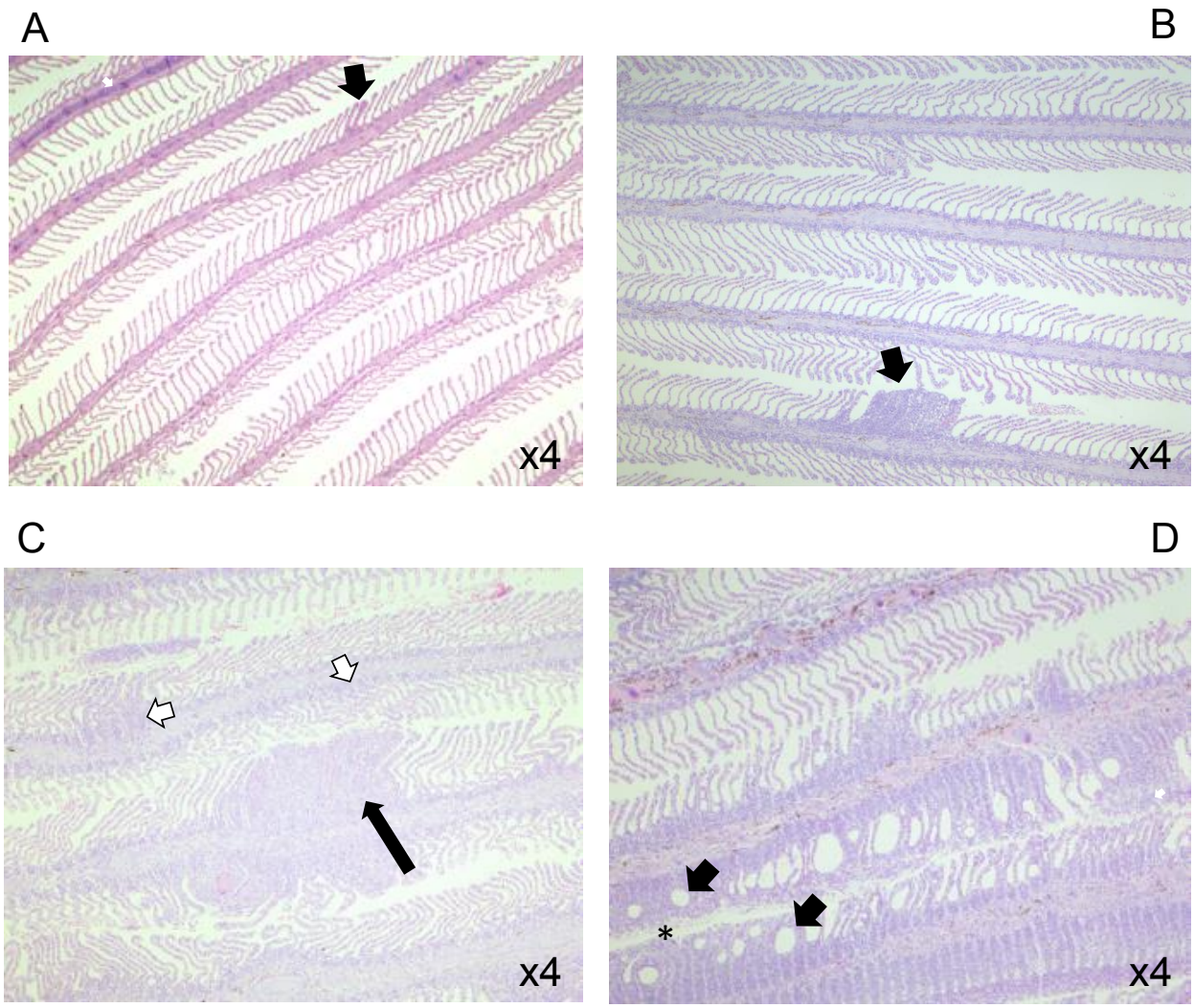


Figure 5.4: *Exemplars of histopathological change in gill tissue, scored using numerical scoring system* (Mitchell et al., 2012). *Sections demonstrating typical gill pathology at varied severity of classification of observed pathology are provided for illustrative purposes. Image A scored between 0-3 and is considered to be largely without pathology. Arrow indicates lamellar clubbing. Image B received an overall score between 4-6, classifying this tissue section as Mildly impacted. Arrow indicates localised lamellar fusion. Image C illustrates gills with a total score between 7-9 classified as Moderate. White arrows indicate hyperplasia with epithelial cell proliferation. Black arrow indicates an example of the multifocal lamellar fusion and hyperplasia observed throughout tissue. Image D shows gill tissue with a score in excess of 10. Black arrows indicate pseudocysts within diffuse, severe structural change to gills, indicative of AGD infection. Asterix highlights presence of an amoebic organism.*

5.4.2.1 Association of histology and gross lesions

Severity of gross pathology and observed histological damage was positively associated, with increased severity of one accompanied by the other. Results were therefore largely in agreement. A number of gills (10) were though observed to suffer histological disease without observation of clinical pathology, and some also had limited histological findings with observed clinical disease. This is likely explainable by the subjectivity in gross assessment and microscopic nature of many structural changes to gill tissue considered pathological. Two fish gill sections were apparently devoid of histological change whilst considered to demonstrate gross clinical change, and while a total of 62 fish were considered to have no major histological change (score 0 – 3), 52 of these fish were considered to have either general or AGD associated gross change.

5.4.3 Adherent microbial communities

The bacterial communities of gills were surveyed by sequencing of gill biopsy samples. Seawater samples were also collected at each sampling exercise and sequenced in addition to spin negative and PCR controls. An average sequencing depth of 10,373 sequences were obtained per gill sample prior to filtering, however a significant proportion of these sequences were unassigned reads. Investigation of these unassigned sequences using BlastN and the Genbank database determined the majority to be of salmonid origin. Following taxa assignation of Amplicon sequence variant (ASVs), followed by filtering of non-bacterial derived sequences, an average of 4275 sequences were obtained per gill sample. A total of 627 bacterial ASV's were obtained from 124 gill tissue samples. These represented 29 phyla, 83 classes, 145 orders, 265 families and 499 genera in total obtained from gill tissue. In addition to the 316 shared with gill samples, a further 366 ASV's were identified uniquely within seawater samples.

5.4.3.1 Gill adherent microbial communities appear distinct from environmental isolates

Despite a number of shared taxa identified, gill samples and concurrently obtained seawater samples from the cage environment appear distinct from each other. Ordination of samples using nmMDS allowed observation of distances between samples within the dataset (**Figure 5.5**), where environmental samples clearly form a distinct cluster from the gill samples. Gill sampling groups appear to be present in loose association (with similar but not identical plotting of concurrently obtained isolates), in chronological order of sampling. Interestingly, early samples appear furthest from seawater-derived populations along the y axis, suggesting the potential for greater similarity of environmental controls with gills of fish that have been longer at sea. Similarity to marine environmental microbial communities appears to increase with duration within the marine environment. Ordination of fish within sampling groups 1 to 11 independently from seawater samples suggests similarity of gill-derived samples to each other. Based on nmMDS visualisation, Groups 1 and 2 appear distinct from other sampling groups, likely due to being so soon after the transfer from freshwater and associated on-farm activities. Remaining samples cluster roughly by sampling group with a large degree of overlap observable.

5.4.3.2 Observation of variable diversity between sampling groups

Diversity indices were obtained and plotted using R to investigate alpha diversity of microbial communities (**Appendix C.3)**. Trends in species richness are apparent across the sampling period, for both evenness and species diversity. Overall taxonomic richness of ASVs appeared to increase following introduction to seawater and initiation of sampling, with a sharp reduction following sampling visit 6. Richness then remained depressed for the remainder of the sampling period. Sampling visits 6 and 7 are conducted only 6 days apart with on farm application of hydrogen peroxide during this brief period between the two samples. Evenness and other indices of diversity vary throughout the sampling period. From these results it is apparent that other environmental or individual factors are having a greater impact on taxonomic richness

within microbial populations beyond merely severity of gill disease. Apparent fluctuations during the sampling period do not appear to be explainable by severity of gill pathology as assessed by histological scoring either. A high level of variation is observed within groups, reflecting the individual variation observed between fish.

5.4.3.3 Dominant phyla across the sampling period

Relative abundance was calculated from filtered sequencing results of each sample to provide information regarding the proportional representation of bacterial genera, families and phyla. When considering isolates at phylum level, a total of 29 phyla were observed with over 95% of observed composition attributable to five phyla in particular. The phyla represented in the majority of samples and cumulatively accounting for >95% of relative ASV abundance in each sample were considered dominant phyla (**Figure 5.6**). These dominant phyla were Bacteroidetes, Proteobacteria, Verrucomicrobia, Firmicutes and Chlamydiae. The abundance of Bacteroidetes, Firmicutes and Chlamydiae vary with sampling group. One-way ANOVA performed in R identified significant differences between groups in abundance of Bacteroidetes ($p = 0.028$), Chlamydiae ($p = 0.00462$), Proteobacteria ($p = 0.000574$) and Verrucomicrobia ($p = 3.73e-10$) during the sampling period. Individual group differences were tested using pairwise t-testing using Benjamini -Hochberg (BH) correction for multiple comparisons (**Figure 5.6)**.

Other identified phyla account for relatively little of the microbial abundance within obtained samples. Of the total number of phyla identified, seven represent taxonomically unclassified bacteria such as BRC1 and TM6, which have been identified in previous next-gen 16S sequencing studies but as yet have not been fully described (Rheims et al., 1996). These poorly classified phyla collectively contribute little to overall estimates of abundance, identified with an average community contribution of < 0.03%. The remaining phyla are well described taxa, present in low abundance.

Chlamydiae abundance is greatest at the start of the study period, in group 1, with overall lowered abundance in subsequent sampling. Trends in Verrucomicrobia

demonstrate an abundance generally lower than 5% in groups, with the exception of Groups 1, 4 and 5. Groups 4 and 5 were identified as having significantly highest Verrucomicrobia community composition. Proteobacteria account for over 80% of abundance in all groups, except for Groups 1 and 5. Trends in abundance of these phyla during the sampling period were considered likely to be explainable by external factors impacting gill bacterial populations, such as on-farm events and concurrent gill tissue variables. The impact of several variables was therefore investigated further.

Figure 5.5 nmMDS of biopsy and environmental samples

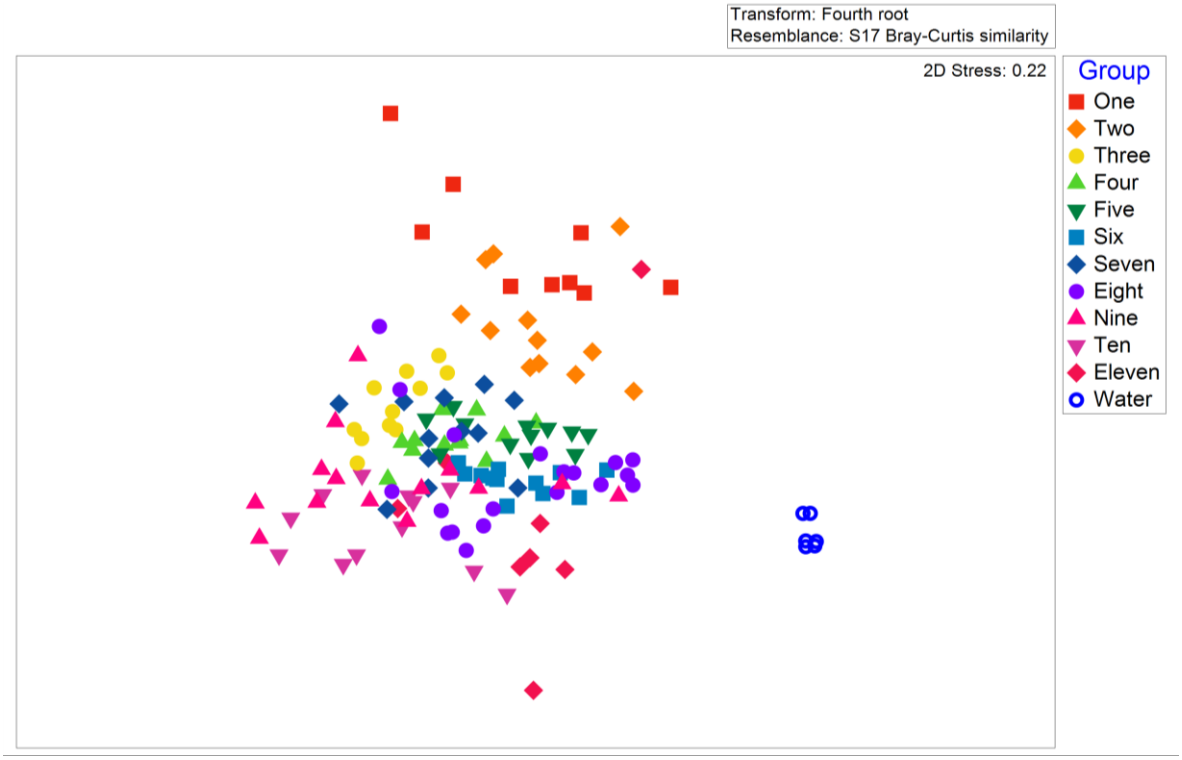


Figure 5.5: *non-metric MDS based on Bray-Curtis similarity matrix of community results at ASV level. Sampling groups 1-11 and environmental isolates are plotted. Groups 1 and 2 can be seen to orientate distally from subsequent gill samples.*

Figure 5.6 Dominant phylum level abundance by group

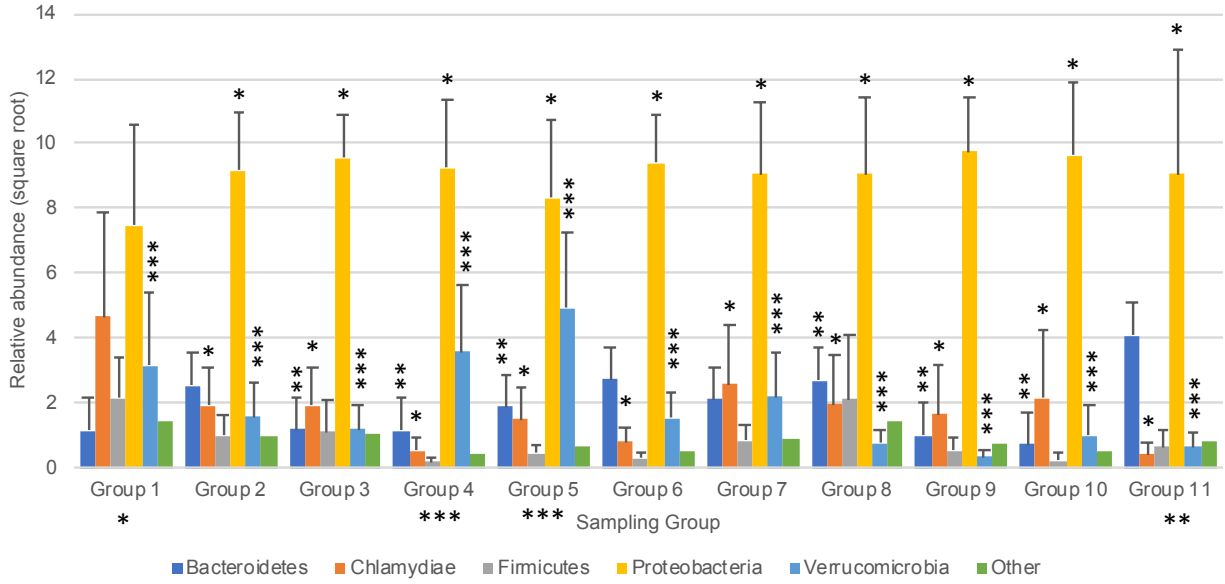


Figure 5.6: *Dominant bacterial phyla across sampling groups. Phyla with average relative abundance of <5% (24 total) have been combined as 'other' to illustrate individually only those phyla with proportionally greatest contribution to bacterial community composition. T-tests with BH corrections for multiple testing for pairwise comparison of dominant phyla levels between groups determined statistically significant differences in dominant phyla of a number of groups. Group one Chlamydiae and Proteobacteria levels are distinct from all other groups (groups with significantly difference abundance to group 1 highlighted above with '*'). Group 11 Bacteroidetes levels are significantly different from a number of other groups (groups with significantly difference abundance to group 11 highlighted above with '**'). Group 5 and 4 Verrucomicrobia levels are distinct from all groups but group 1 (groups with significantly difference abundance to groups 4 and 5 are highlighted above with '***')*

5.4.3.4 High variation between individuals within sampling groups

A large degree of variation was observed between concurrently obtained individual samples. Visualisation using the commonly used stacked bar graph greatly misrepresented results through utilisation of a group average. Due to the masking of high and low abundance in shared taxa within sampling groups this was therefore not considered an appropriate visual representation of the data. Results are instead

presented for each individual fish gill sample in **Figure 5.7** (at class and phylum level), alongside results of gross and histological pathology. Appraisal of individual samples identifies fish with abundance of over 92% for some taxa concurrently identified with abundance of less than 15% in other individuals within the same sampling group. However, a number of classes were observed with high relative abundance across individuals within groups, including Chlamydiae, Actinobacteriae, Flavobacterae and Verrucomicrobia, as well as Alpha, Beta and Gammaproteobacteria. General trends in abundance across groups appear to demonstrate a lower abundance of Betaproteobacteria and greater abundance of Gammaproteobacteria in initial sampling groups, with increased Betaproteobacterial abundance throughout the study, and reduced abundance of Gammaproteobacteria in later samples. Abundance of Bacilli and Clostridia appears to vary across groups, as do Flavobacteria and Verrucomicrobia. Abundance of other classes appeared to be generally low, with high abundance in a small number of individuals in separate groups. Despite overall identification of a large number of taxonomically distinct bacterial phyla and classes, the majority of taxa were identified in relatively few samples. This low number of shared taxa might represent a core microbial community of gill tissue. Beyond these shared taxa, high individual variation is apparent. This variation might be considered to be in the transient bacterial populations of the microbial community, with high apparent abundance of many taxa driven by as yet undetermined variables. Whilst environmental factors are known to impact microbial community composition, variation even within groups experiencing identical conditions suggests involvement of additional, individual variables. Host genetics and disease state were considered potential driving factors of this variation.

5.4.4 Gill disease as a potential explanatory variable for microbial community variation between individuals

Previous research shows a high degree of variation in gill-associated microbiomes between individual fish (Boutin et al., 2014), however there is no clear identification of potential explanatory factors. Obviously, fish are individuals and as such natural variation will occur. However, salmonids (particularly those in the farmed environment), are exposed to a number of severe environmental challenges during their

life cycle. Farmed fish in particular suffer gill pathologies in the marine environment, with fish in this study observed to be suffering varying level of disease within sample groups. Results of the histological scoring system utilised are illustrated alongside microbial abundance results in **Figure 5.7**. Through this combined visualisation, associated patterns of altered abundance appear to correlate with observed gill pathology, particularly in Groups 4 and 5. Increased Verrucomicrobia abundance can be observed, alongside what appears to be a reduced abundance of Bacteroidetes and Chlamydiae families. Gill disease was therefore investigated as a potential explanatory variable for shifts in relative microbial abundance between samples. Use of a previously described, semi-quantitative gill scoring methodology allowed characterisation of gills with different levels of pathology (Mitchell et al., 2012). This scoring system allowed generalised classification of gill sections as none, mild, moderate or severe through collective assessment of multiple and varied indicators of disease. The cause of pathology was not definitively determined. Instead, gills were considered to be experiencing multiple insults with a combined impact on overall health.

5.4.4.1 Association of samples by severity of histological gill disease

Cluster analysis applied to entire sample sets demonstrates an association of samples largely by sample collection group (**Figure 5.8**). Two-way PERMANOVA analysis identified sampling group as a statistically significant predictor of microbial results (pseudo-F = 3.684, p = 0.001), with subsequent ANOSIM testing identifying statistically significant differences between the community composition of individual sampling groups (**Appendix C.5**). Significant community-wide differences were not detected between fish grouped by severity of gill lesions in two-way PERMANOVA analysis (pseudo-F 1.878, p = 0.441). This result is supported by the performance of redundancy analysis (RDA), that suggests greatest variation is explainable by sampling group, but suggests variation is impacted too by histological scoring, with limited collinearity (**Appendix C.5**). Incase results of statistical analysis were impacted by relative lack of samples in fish considered severely impacted (n = 4), analyses were repeated with reclassification of gill disease as either ‘healthy’ (no or only mild pathology) or ‘diseased’ (moderate and severe pathology). Repeat PERMANOVA with

this new differentiation does then suggest community-wide differences in results (pseudo-F = 1.6202, p = 0.019), albeit still not significant statistically. PERMANOVA results therefore suggest that timing of sample collection (i.e., group number) is the most important factor in beta diversity, and two-way testing identified no interaction between these independent variables (pseudo-F = 0.9997, p = 0.479). Variation between groups is likely driven by a number of factors, including environmental parameters, seasonal trends and on-farm events.

PCA analysis (**Figure 5.8**) demonstrates a trend in sample ordination by severity of gill histology. A number of principal components contribute to similar proportions of variation within the dataset, however PC1 and PC2 together account for the greatest variation (44.1%) and so were utilised in construction of the PCA plots in **Figure 5.8**. PC1 appears to be at least partially explainable by sampling date, with results of groups 1 and 2 clustering relatively distant from the remaining groups. Beside groups 1 and 2, a large degree of superimposition is apparent for the remaining groups, suggesting impact of additional factors beyond timing of sampling. When results are visualised by severity of gill disease, it is apparent that individuals separate along PC2 by severity of gill pathology. Fish determined to have severe and moderate histological classification separate from those considered to be experiencing no notable pathology. PCA plots therefore suggest beta diversity might be impacted by the severity of gill pathology, as determined by histological score. Principal component analysis of gill biopsy samples (**Figure 5.8**) relative to the observed histological gill pathology demonstrates then apparent separation by severity of gill lesion along PC2. It would therefore seem that despite no overall statistically significant variation in community composition, the factors contributing to PC2 may be of importance in driving observed community differences between different degrees of gill pathology.

Figure 5.7 : Class level trends during sampling duration

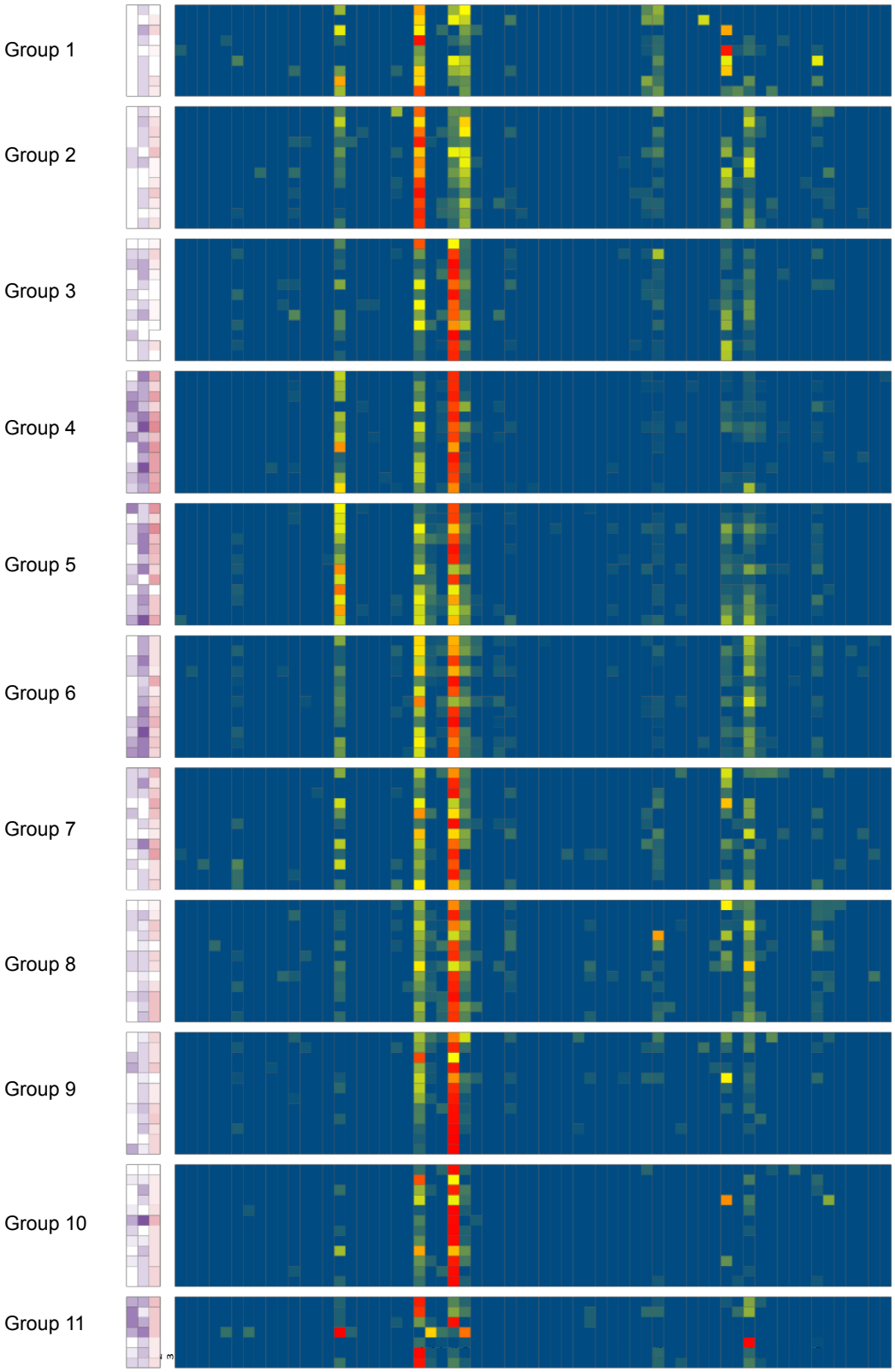


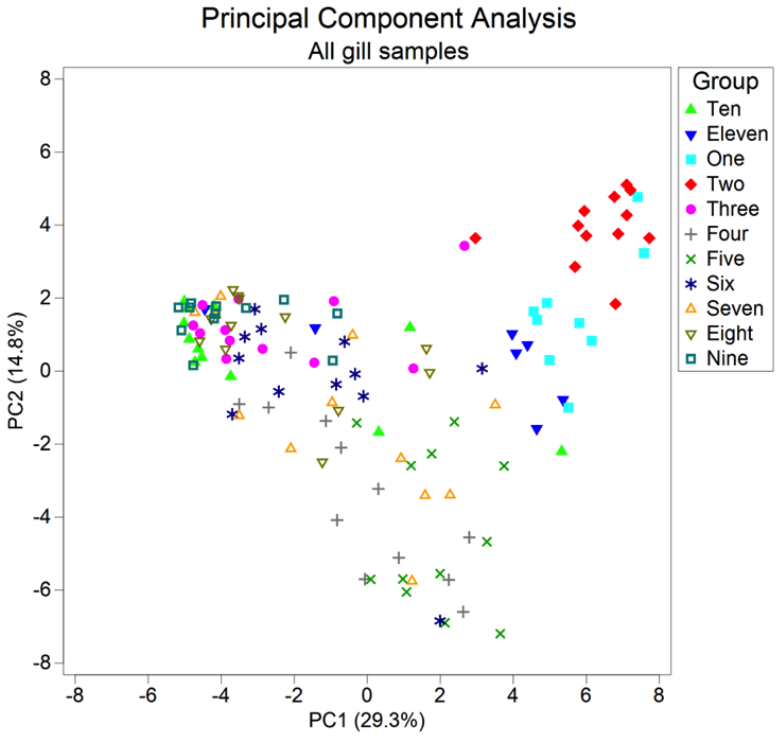
Table 5.1 Key for Class-level abundance figure

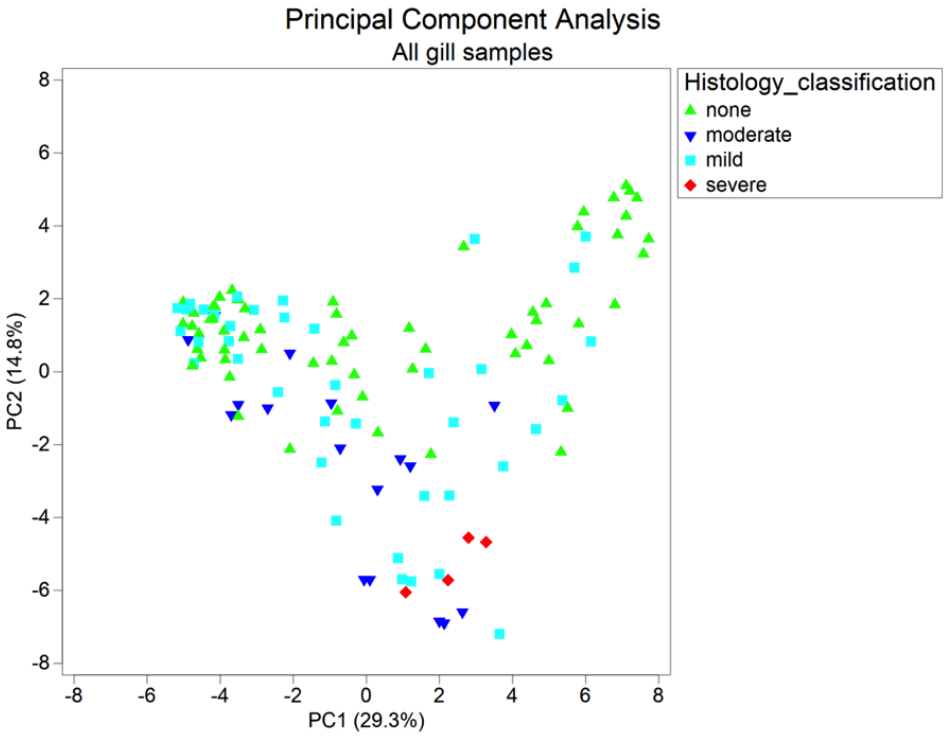
Bacteria	Acidobacteria	Holophagae	2
		Solibacteres	3
		Subgroup 17	4
		Subgroup 6	5
	Actinobacteria	Acidimicrobiia	6
		Actinobacteria	7
		Thermoleophilia	8
	Bacteroidetes	Ambiguous taxa	9
		Bacteroidetes Incertae Sedis	10
		Bacteroidia	11
		Cytophagia	12
		Flavobacteriia	13
		Sphingobacteriia	14
	Chlamydiae	Chlamydiae	15
	Chloroflexi	Ambiguous taxa	16
		Anaerolineae	17
		Caldilineae	18
	Deinococcus-Thermus	Deinococci	19
	Elusimicrobia	Elusimicrobia	20
	Firmicutes	Bacilli	21
		Clostridia	22
		Erysipelotrichia	23
		Negativicutes	24
	Fusobacteria	Fusobacteriia	25
	Gemmatimonadetes	Ambigous taxa	26
	Gracilibacteria	Ambiguous taxa	27
		Gyrodactylus salaris	28
	Latescibacteria	Ambigous taxa	29
	Lentisphaerae	Lentisphaeria	30
	Marinimicrobia	Ambigous taxa	31
	Nitrospinae	Ambigous taxa	32
	Omnitrophica	Omnitrophica Incertae Sedis	33
	Parcubacteria	Ambiguous taxa	34
	Planctomycetes	Ambiguous taxa	35
		Phycisphaerae	36
		Planctomycetacia	37
	Proteobacteria	Alphaproteobacteria	38
		Betaproteobacteria	39
		Deltaproteobacteria	40
		Epsilonproteobacteria	41
		Gammaproteobacteria	42
	Saccharibacteria	Ambiguous taxa	43
	Tenericutes	Ambiguous taxa	44
	Verrucomicrobia	Mollicutes	45
		Ambiguous taxa	46
		Opitutae	47
		Spartobacteria	48
		Verrucomicrobiae	49
	Unclassified	Candidatus Daviesbacteria	50
		Candidatus Roizmanbacteria	51
		Candidatus Adlerbacteria	52
		Candidatus Azambacteria	53
		Candidatus Campbellbacteria	54
		Candidatus Falkowbacteria	55
		Candidatus Magasanikbacteria	56
		Candidatus Moranbacteria	57
		Candidatus Nomurabacteria	58
		Candidatus Berkelbacteria	59
		RBG-1 (Zixibacteria)	60
		SR1 (Absconditabacteria)	61
		BRC1	62
		TM6 (Dependentiae)	63

Figure 5.7 + Table 5.1*: Chart represents microbial abundance on a blue to red spectrum, with blue being absent taxa and red being highly abundance. Each box represents a class of microbe present within the dataset, corresponding to numbers 1 – 63. To the left, histological score is illustrated by intensity of red, and gross pathological scores by intensity of purple boxes. Table 1 provides taxonomic information for numbers columns.*

A

Figure 5.8 : PCA and hierarchical clustering by severity of disease





B

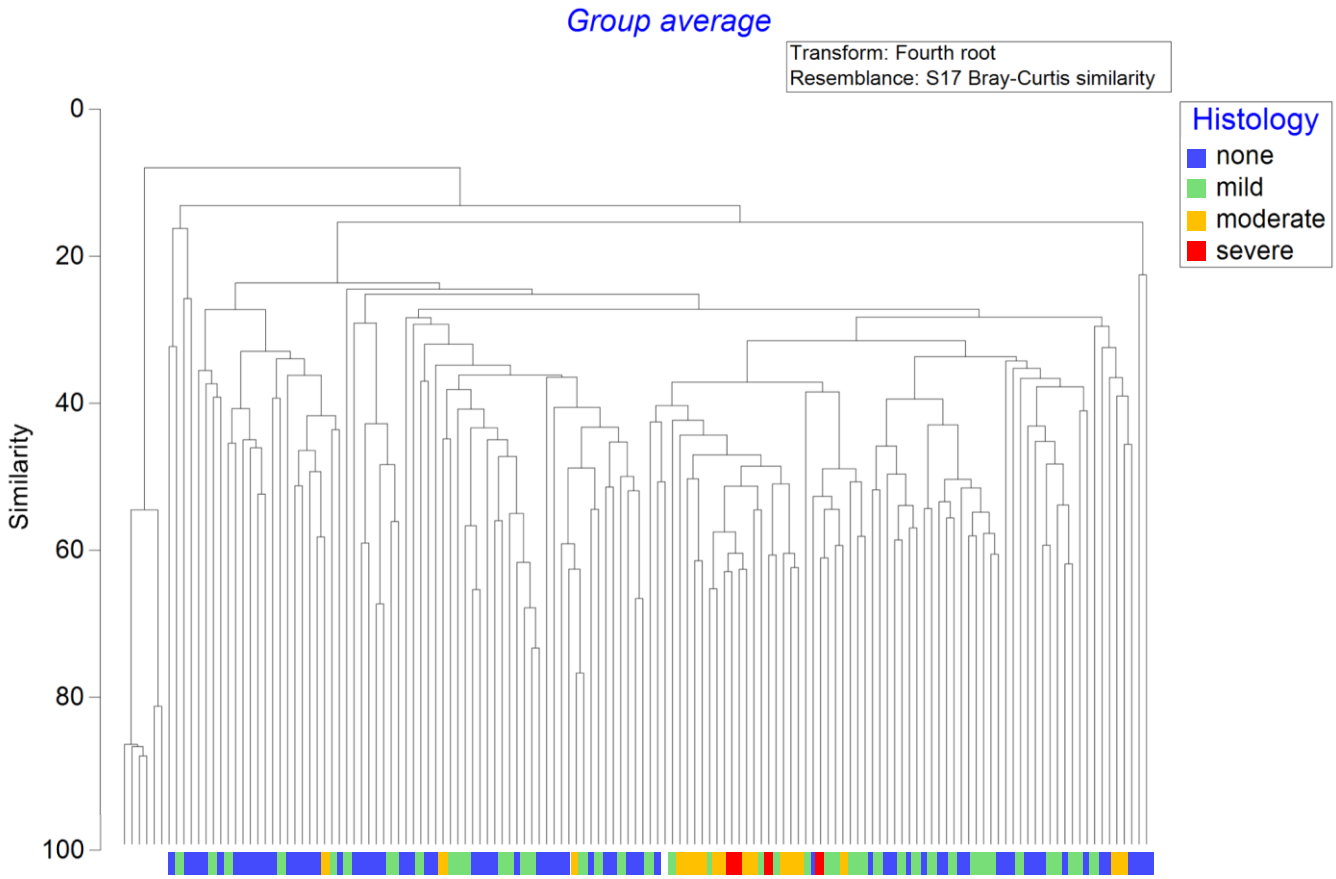


Figure 5.8 A & B. *A: Identical PCA construction with samples coloured by sample group (left) and gill pathology (right), as determined by histological score. PCA suggests beta diversity might be impacted by severity of gill pathology, supported by the hierarchical cluster analysis (B) of samples, based on Bray-Curtis similarity matrix of all sample's, groups 1 – 11, with association of Moderate and Severely impacted samples together.*

5.4.4.2 Proportional change in microbial taxa with observed severity of histological lesion

PCA-derived loading values were obtained to identify taxa with the greatest proportional contribution to observed variance. The taxa, classified to genus level, with the greatest contribution to PC1 and PC2 are shown in **Figure 5.9**. These include taxonomically ambiguous genera within the families *Procabacteriaceae* and *Flavobacterium*, as well as the genera *Shewanella, Rubritalea, Serratia, Psychrobacter,*

Pseudoalteromonas and *Pseudomonas* genera. Three as-yet taxonomically ambiguous microbes were also identified as having high proportional contribution to PC2; *Candidatus Branchiomonas, Candidatus Fritschea* and *Candidatus Piscichlamydia*.

Whilst comparisons of entire microbial communities between individual fish gills by PERMANOVA identified no statistically significant differences between fish relative to severity of histological pathology, paired t-testing with BH correction for multiple comparisons did identify significant differences in abundance of the above taxa between gill pathologies (**Figure 5.10 to Figure 5.13**). Results of PCA suggest a small number of bacteria are having a relatively large proportional impact on the variance that is observed within multivariate analyses. These results are supported by the results of SIMPER analysis, used to determine the taxa with greatest variation between histologically classified gill tissue samples (**Appendix C.6).** Taxa highlighted in SIMPER analysis largely mirror taxa observed within PC2. Taken individually, SIMPER analysis lacks the ability to appraise individual samples, and can mask large standard deviations in proportional representation. However, supported by the results of PCA, the bacterial taxa identified in these analyses may be the drivers of gill pathology variation that can be observed between gill samples in this study.

Relative abundance of the above microbial taxa identified as being of interest in explaining variation between different disease states of gills were plotted. Those taxa identified as having significant variation with gill pathology are illustrated in **Figure 5.10 – 5.13**. Apparent association with increasing severity of gill disease is observed for *Candidatus Branchiomonas, Candidatus Fritschea* and *Rubritalea. Procabacteriaceae* abundance appears to be associated with gill health as well, however unlike the other identified microbes, abundance of this taxon is greatest in healthier gill tissue.

Figure 5.9 Loading values PC1 and PC2

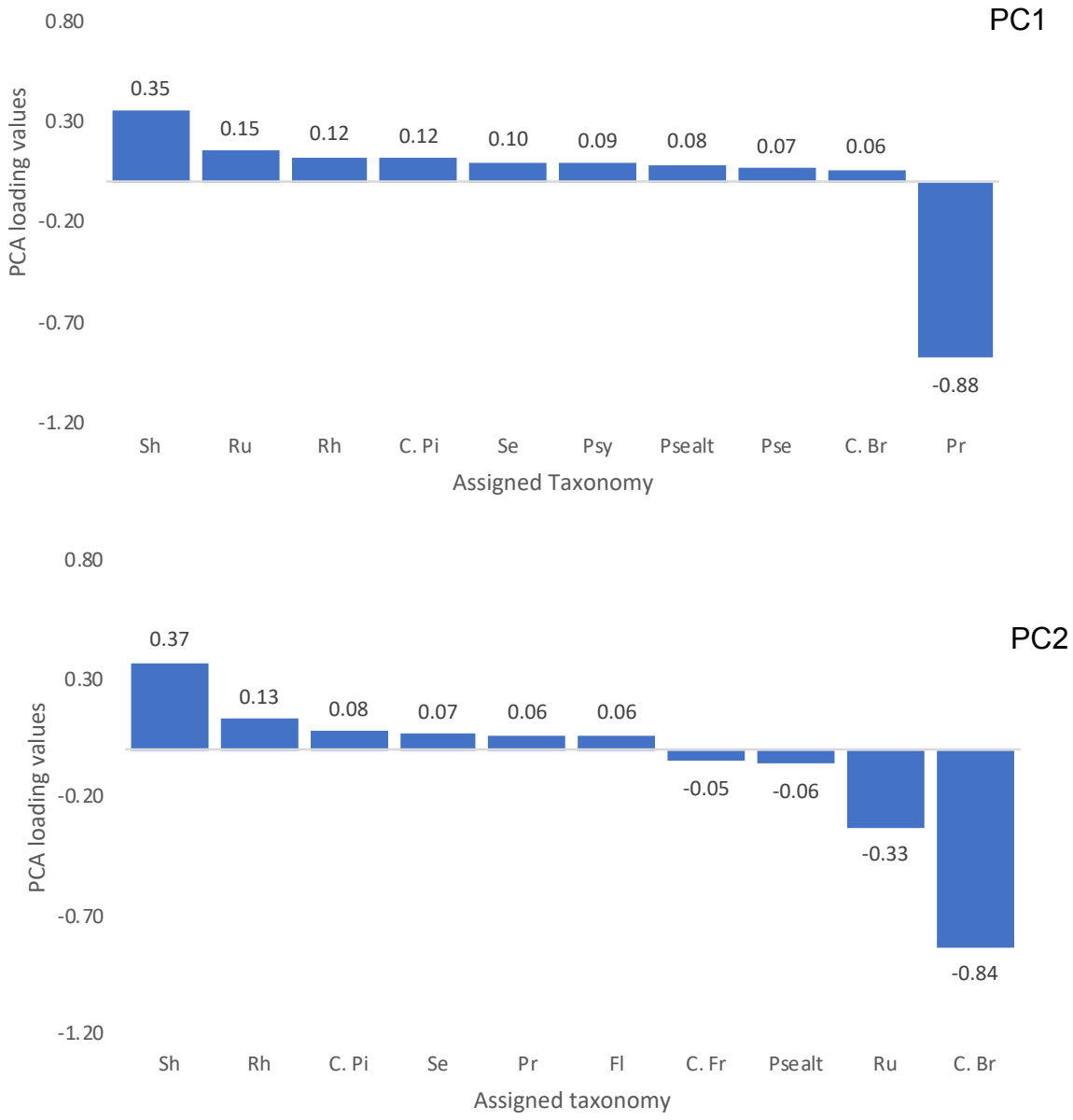
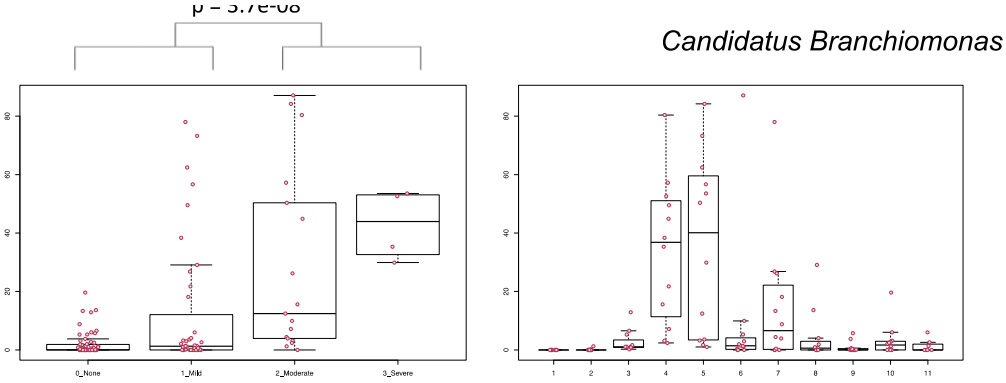


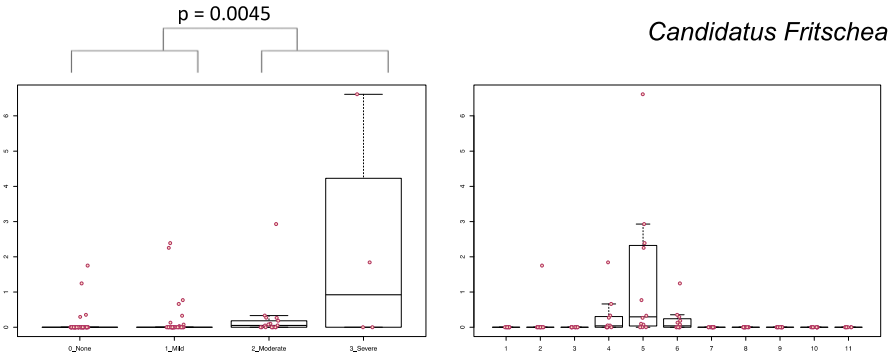
Figure 5.9. *Loading values with proportionally greatest contribution to principal components 1 and 2. Pr = Procabacteriaceae, Sh = Shewanella, Ru = Rubritalea, Rh = Rhodobacteraceae; unknown species, C. Pi = Candidatus Piscichlamydia, Se = Serratia, Psy = Psychrobacter, Psealt = Pseudoalteromonas, Pse = Pseudomonas, C. Br = Candidatus Branchiomonas, C. Fr = Candidatus Fritschea, Fl = Flavobacterium.*

Figure 5.10 *Candidatus Branchiomonas* abundance



	None	Mild	Moderate
Mild	**0.0058**	-	**0.002**
Moderate	**8.50E-07**	**0.002**	-
Severe	**4.40E-05**	**0.002**	0.1533

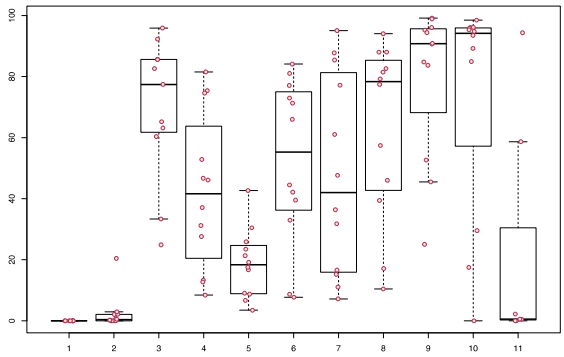
Figure 5.11 *Candidatus Fritschea* abundance



	None	Mild	Moderate
Mild	0.51	-	0.66
Moderate	0.44	0.66	-
Severe	**1.30E-07**	**4.40E-07**	**3.30E-06**

Figure 5.12 *Procabacteriaceae* abundance

Procabacteriaceae



	Eight	Eleven	Five	Four	Nine	One	Seven	Six	Ten	Three
Eleven	**0.00233**	-	-	-	-	-	-	-	-	-
Five	**0.00013**	0.7839	-	-	-	-	-	-	-	-
Four	0.06841	0.14275	**0.04437**	-	-	-	-	-	-	-
Nine	0.15357	**2.80E-05**	**3.30E-07**	0.00121	-	-	-	-	-	-
One	**7.90E-07**	0.12663	0.14194	0.00071	**3.50E-09**	-	-	-	-	-
Seven	0.16711	0.06275	**0.01307**	0.65234	**0.00568**	**0.00014**	-	-	-	-
Six	0.35306	**0.02644**	**0.00381**	0.39459	**0.01902**	**3.60E-05**	0.69133	-	-	-
Ten	0.35896	**0.00013**	**2.80E-06**	**0.00568**	0.65234	**1.90E-08**	**0.02214**	**0.06233**	-	-
Three	0.62798	**0.00059**	**2.50E-05**	**0.02176**	0.39629	**1.60E-07**	**0.06512**	0.14275	0.69133	-
Two	**3.30E-07**	0.14275	0.15357	**0.00059**	**1.30E-09**	0.8284	**0.00011**	**2.50E-05**	**6.10E-09**	**6.20E-08**

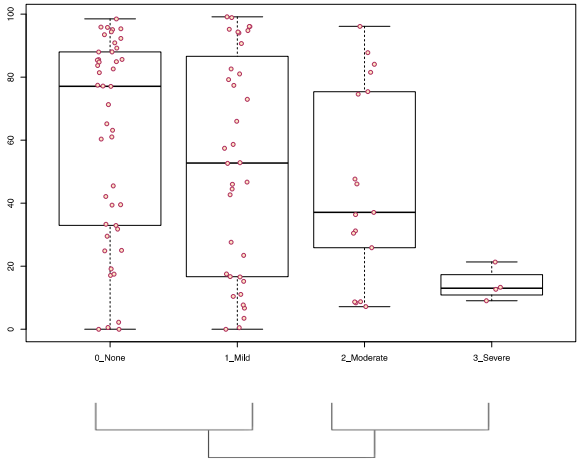
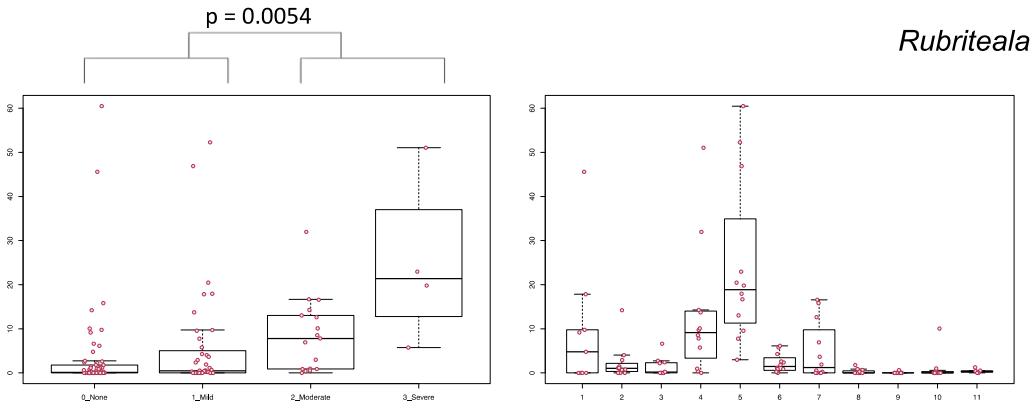


Figure 5.13 *Rubritalea* abundance



	None	Mild	Moderate
Mild	0.34669	-	0.34715
Moderate	0.11465	0.34715	-
Severe	**0.00085**	**0.00228**	**0.01303**

Figures 5.10 – 5.13*: Boxplots illustrating the trends in altered average abundance by histological classification of gills, as well as by sampling group. P values for comparisons between fish classified as 'healthy' (none and mild pathology) to those considered 'diseased' (moderate and severe pathology) are illustrated above plots. Figures representing the BH corrected p values of comparisons between all histological presentation groups are provided also in tables below the figure.*

5.4.4.3 Lack of correlation of diversity with histological gill disease

Analysis of species richness, diversity and evenness across severity of gill presentations identified no clear trends (**Appendix C.3**). These analyses were again probably hindered by the low number of individuals considered to be experiencing severe gill trauma (n = 4) relative to those with none and mild disease. Repeat analysis again with reclassified 'healthy' and 'diseased' histological categories (not shown) also demonstrated no significant differences. Based on these results, the factors with

arguably the greatest impact on microbial diversity and richness are not disease-associated, but instead they are likely to be environmental, seasonal and on-farm treatments of fish, the impacts of which can be more easily explained by association of samples by collection group rather than severity of gill disease.

5.4.4.4 Further work in determining trends between 'healthy' and 'diseased' gill tissue

Previous research has utilised proportions of phyla within groups (expressed as a ratio) as a metric for assessing alterations to microbial communities in human disease states, identifying Firmicute and Bacteroidetes ratios as important predictors of nutritional disease (Bervoets et al., 2013; Riva et al., 2017). Significant differences in the ratio of compartment-specific communities has been demonstrated in fish as well, although this research was not able to differentiate 'healthy' and 'diseased' fish through comparisons of samples originating from the same tissue (Legrand et al., 2018). Application of this technique to these results was performed to obtain ratios of relative abundance between the dominant phyla within samples, as defined by the phyla constituting the greatest relative abundance (>0.5%). These relative ratios of phyla between groups and different categories of gill pathology were plotted for each combination of dominant phyla, allowing observation of trends between groups (not shown). These comparisons across sampling groups appeared to support the observable trends in **Figure 5.7**, with shifts in dominant phyla between sampling visits. Relative ratios appear altered between groups known to contain fish with greatest severity of gill pathology.

When fish were grouped by histological score, application of Welch's paired t-test to plots of relative ratios of phyla was performed to determine if any of the apparent shifts with gill disease might be considered statistically significant. Ratio of Verrucomicrobia relative to other dominant phyla were investigated based on the significant proportional increase of this phylum in severely impacted gills alongside reduction in abundance of other phyla, and apparent variation in ratio to other phyla for groups 4 and 5 (**Figure 5.14**). Initial pairwise t-test results (corrected for multiple testing using the Benjamini-Hochberg method) were performed to compare gills classified as having none, mild, moderate and severe gill pathology. These results suggest that no significant differences

exist in comparison of any groups, however they suffer again from the small sample size of severely impacted gills. When gills are reclassified as ‘healthy’ (none and mild groups) and ‘diseased’ (moderate and severe) and compared using one-way ANOVA, a significant difference was detected in the Bacteroidete:Verrucomicrobia ratios of these groups ($p = 0.0419$). Thus, there appears a significant predominance of Verrucomicrobia relative to Bacteroidetes in more heavily diseased gills.

Figure 5.14 Phyla ratios

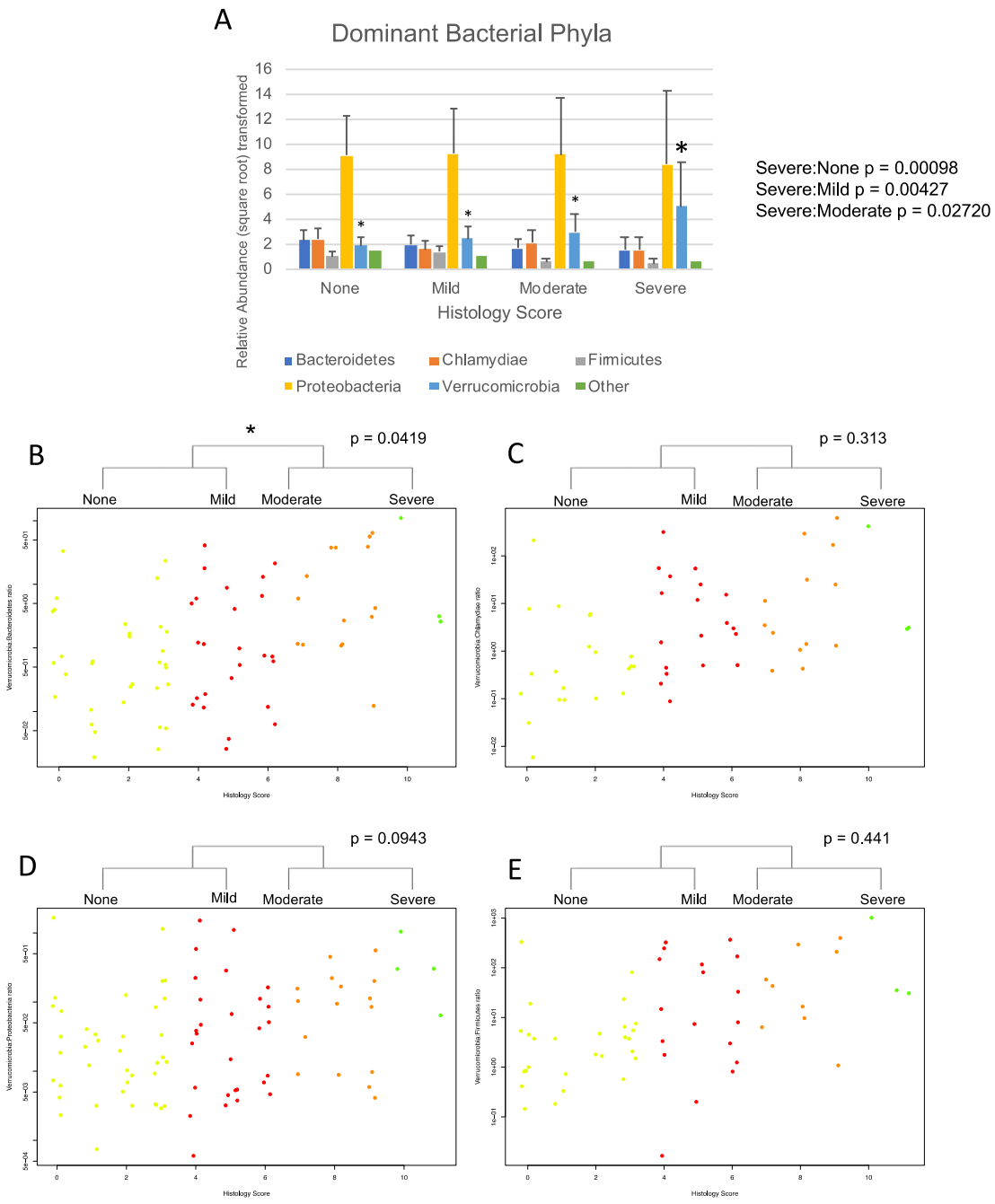


Figure 5.14: *A comparison of Verrucomicrobia to other dominant phyla present within gill samples. Relative abundance of phyla is presented for each gill histological classification (A), alongside relative ratios of Verrucomicrobia to other dominant phyla (B, C, D and E). Significant variation is indicated by an Asterix (*).*

5.4.5 External drivers of observed variation in microbial community composition

5.4.5.1 Hydrogen peroxide

Hydrogen peroxide (H_2O_2) is used in the farm situation to treat a number of diseases. Fish in this study were treated by SSF operatives between sampling visits 6 and 7 for control of the sea lice parasite. Samples were collected only 6 days apart, with application of hydrogen peroxide performed 4 days after collection of sample 6, and 2 days prior to sample 7 collection. Whilst earlier investigation of various diversity measures identified no association of severity of gill pathology with alterations in measured indices, species richness plotted by sample group does suggest a dramatic alteration in community structure between groups 6 and 7. Earlier analysis of overall beta diversity of community composition between these groups using ANOSIM also suggested significant differences in community composition between these groups ($p = 0.001\%$). (**Appendix C.4).** Hierarchical clustering and PCoA analysis were therefore performed of groups 6 and 7 alone (**Figure 5.15**), with results demonstrating clear separation of samples by treatment group. These results strongly support a significant impact on the microbial community composition of gill tissue by application of alkaline veterinary medicine, H_2O_2.

Individual microbial differences are observed too between groups 6 and 7 at various taxonomic levels. Relative ratios of dominant phyla between sampling groups 6 and 7 demonstrated an apparent change to the relative ratio of Bacteroidetes and Firmicutes (**Appendix C.7**). Statistical testing of the differences between relative ratios of groups using Benjamini-Hochberg corrected pairwise t-testing identifies a significant difference between these groups ($p = 0.00023$). Changes to adherent taxa present in the majority of samples (>10 individuals) within a particular group following treatment with H_2O_2 demonstrates quite different results in groups 6 and 7 at the genus level too (**Table 5.2).** Genera identified in the majority of samples prior to H_2O_2 application appear to be much rarer in samples following treatment. When they are identified, the apparent abundance of these taxa also appears generally reduced within fish sampled after H_2O_2 treatment. For example, *Flavobacterium* and *Candidatus Piscichlamydia* are

considered to have significantly changed in their relative abundance between these two groups. Following application of H_2O_2, it appears as though individual variation is increased, as commonly identified microbiota (present in >10 samples within a group) are reduced or lost from adherent populations and replaced with a more variable microbial community. Only Serratia appear to increase in the number of individuals they are observed within following treatment of the populations with H_2O_2.

Figure 5.15 Pre and post hydrogen peroxide communities

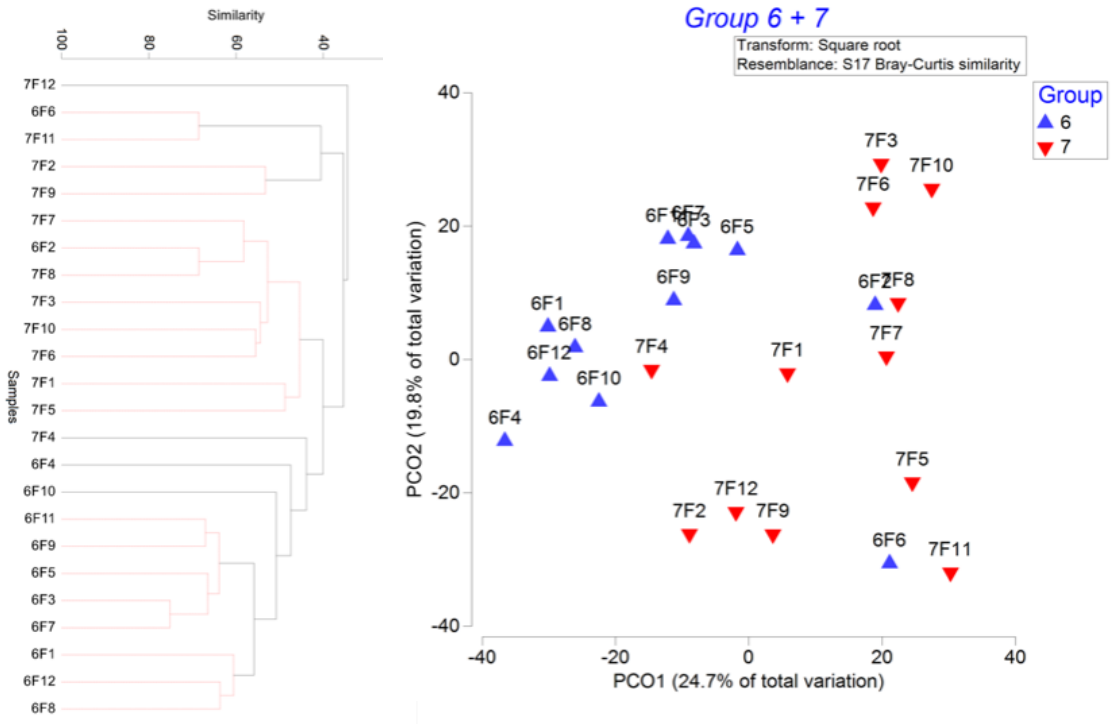


Figure 5.15: *Hierarchical cluster analysis and PCoA of groups 6 and 7 independently from the rest of the dataset. Figures were constructed as previously detailed, using software Primer 7 and Bray-Curtis similarity matrices. Samples can be seen to orientate clearly with those from the same sampling group, despite collection only days apart. Sample are not from the same individuals and names are provided only to make appraisal of results in both figures more accessible. It is apparent that an alteration to the microbial community is occurring in these gills.*

Table 5.2 Before and after hydrogen peroxide

Phylum	Class	Order	Family	Genus	Abundance		Sample Representation		p
					Pre-H2O2	Post H2O2	Pre-H2O2	Post-H2O2	
Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	*Chryseobacterium*	0.89 ± 0.89	0.94 ± 5.16	12	5	0.0
	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	*Flavobacterium*	4.13 ± 3.67	0.63 ± 3.98	12	4	0.0
	Flavobacteriia	Flavobacteriales	Flavobacteriaceae		0.74 ± 1.12	0.89 ± 6.66	11	4	0.0
	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	*Tenacibaculum*	0.35 ± 0.39	1.25 ± 5.77	9	5	0.1
	Cytophagia	Cytophagales	Cytophagaceae	*Hymenobacter*	0.49 ± 0.54	0.11 ± 1.16	10	1	0.0
Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Rubritaleaceae	*Rubritalea*	2.20 ± 2.32	4.86 ± 11.7	11	8	0.0
Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae		0.42 ± 0.52	0.09 ± 0.63	9	2	0.3
	Betaproteobacteria	Procabacteriales	Procabacteriaceae		52.33 ±	47.7 ± 47.4	12	12	0.9
	Betaproteobacteria	Nitrosomonadales	Nitrosomonadaceae	*Candidatus Branchiomonas*	9.31 ± 32	15 ±	9	9	0.1
	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	*Acinetobacter*	0.25 ± 0.21	0.53 ±	9	7	
	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	*Psychrobacter*	15.78 ± 14.1	7.46 ± 24	12	11	0.1
	Gammaproteobacteria	Alteromonadales	Pseudoalteromonadaceae	*Pseudoalteromonas*	2.87 ± 2.29	1.33 ± 11.8	12	3	0.0
	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Serratia*	0.51 ± 0.77	2.39 ± 4.79	11	12	1E-
Chlamydiae	Chlamydiae	Chlamydiales	Chlamydiales	*Candidatus Piscichlamydia*	0.43 ± 0.66	6.53 ±	9	9	0.0

Table 5.2*: Genus level analysis of variation between H_2O_2 untreated and treated gills. Genera represented in the majority of individuals pre-treatment (ten or more fish) are included to illustrate the altered presence within the population following treatment with H_2O_2. P values are obtained by one-way ANOVA of groups of fish pre and post H_2O_2 treatment.*

5.5 Discussion

A large dataset of information regarding the microbiota of Atlantic salmon gills was obtained by the work described in this chapter, charting fish from introduction to seawater to near harvest size. The of aim investigating the variation of the microbiota between individuals with different levels of gill pathology was explored alongside a number of additional variables that might be drivers of varied community composition. The association of gill pathology with varied community composition throughout the dataset was explored, as well the differences between groups sampled before and after hydrogen peroxide treatment (H_2O_2). Results highlight high individual variability between fish, potentially partially due to gill disease. However, other environmental and undetected variables in the marine environment appear to also have significantly impacted the dataset. Overall, results provide valuable insight into the microbial communities of farmed Atlantic salmon during the marine phase of production. Interesting findings were obtained regarding microbial community structure with varied severity of gill disease, as well as the influence of hydrogen peroxide treatment during production.

5.5.1 Experimental design and laboratory work

5.5.1.1 Sample handling and DNA extraction

Issues were encountered during this research with regards extracting microbial DNA from fish gills. For example, apparent inhibition of initial PCRs was encountered, determined to be due to protein contamination. The DNeasy Blood + Tissue extraction kit (Qiagen) used recommends a lower tissue weight when used on splenic tissue due to the highly perfused nature of the spleen, with large numbers of red blood cells (RBCs). Gills are also highly perfused to facilitate gas exchange at their surface, and so in addition to the protein-containing gill structure and proteinaceous mucus on the gill surface, samples might contain a large amount of protein in the form of haemoglobin (contained within RBCs). As fish were not bled prior to removal of gill sections due to the time pressure for tissue removal before induction of autolysis in gills, RBCs

remained within the gills, potentially increasing protein contamination and impairing the activity of Taq (Schrader et al., 2012). Modification of the protocol for digestion of tissue sections at the low end of the recommended range (average 10 mg) and introduction of phenol/chloroform step resolved this issue. Although these are small sections, the surface area of gills is functionally required to be large, with a large area theoretically capable of harbouring a greater number of bacteria when compared to sections of similar weight from tissue such as skin. Bleeding fish from the tail was not considered due to the early onset of autolysis in gill tissue, however it might have improved DNA extraction results and reduced inclusion of salmonid material in results. Bleeding fish from the gills would not have been appropriate for this research as intact gill tissue was one of the goals of the sampling.

Whilst there exists a number of papers detailing the microbiota of gill and skin tissue, the majority utilize a method of swabbing the gill surface to obtain samples. However of interest particularly in this study were pathogenic microbes that might be located intracellularly or within the tissue structure (Nowak and LaPatra, 2006).
Previous literature regarding the presence of bacteria, using scanning electron microscopy, suggest that many of the adherent microbiota can be cryptically located (Mudarris and Austin, 1988), with the structure of gills potentially preventing access to these populations from swabbing alone. Biopsies were therefore utilised to obtain the results of this chapter as it was suspected that swabbing might fail to capture populations of bacteria between filaments or present as part of infective lesions. In hindsight, biopsies introduced a number of issues in laboratory processing and data collection as part of this research.

5.5.1.2 Next generation sequencing

Modification of the protocol for sequencing samples included an increase in PCR cycles from 25 to 27 during the amplification step. Whilst this was designed to maximise PCR product output, it has the potential to have altered results. Firmicutes and Gammaproteobacteria in particular are known to have a high 16S copy count (Větrovský and Baldrian, 2013), a fact that might alter community abundance estimates

with increase PCR cycles, magnifying this difference in apparent abundance, making it appear as though these microbes appear more proportionally dominant in results. Although this bias was certainly a potential factor in this research, identical treatment and PCR of all samples ensured at least an identical bias, so fluctuations in relative abundance between groups remains meaningful. In hindsight, the use of the V3/4 region allowed amplification of a high proportion of host DNA, which proved problematic in sequencing. Masking of host DNA might have been minimised by introduction of blocking agents or other methods of removing host DNA prior to next generation sequencing.

5.5.2 A distinct gill tissue microbial community

5.5.2.1 Divergence from environmental communities

Beta diversity results (**Figure 5.5**) demonstrate dissimilarity between gill-associated and environmental microbial communities. This is in agreement with previous research that identifies significant differences between salmonid skin and gill-associated microbiota and that of the surrounding seawater (Brown et al., 2019). The microbiota of Atlantic salmon gills are thought to be distinct from environmental populations due to modulation by the existing microbial community and host innate immune adaptations (Honda and Littman, 2012; Minniti et al., 2017). These previous conclusions are mirrored in the results of this chapter. Ordination of samples by nmMDS also demonstrates an arguably more distinct separation along the y axis of samples in groups 1 and 2 from seawater samples, with samples plotted in apparent isolation from subsequent gill samples. Fish were introduced to saltwater almost immediately prior to initiation of the sampling period, with only three weeks of marine adaptation prior to collection of gills from sample group 1. It therefore appears from results that microbial shifts on the gill surface, previously demonstrated to occur immediately following entry to seawater (Lokesh and Kirin, 2016), actually continue to occur for some time afterwards as well, with group 2 sampled nearly two months after introduction to seawater. Confounding factors to this conclusion though might include a diet change in the marine environment.

Regardless of this, these results show that microbial communities continue to alter for an extended time period after entry to the marine environment. Community composition of the sample groups beyond sample 3 appear to be less dissimilar, suggesting a level of homeostasis was achieved in the gill microbiome of this population around this time (**Figure 5.5**). Later samples appear more similar in community composition to environmental samples with less distinction between groups 3 onwards than samples from the first sampling groups. Group differences are however still detected throughout the sampling period (**Appendix C.4**). The microbial community of earlier groups likely contains more freshwater-derived bacteria, with restructuring of the gill microbiota in later sample groups from incorporation of marine-derived bacteria explaining the increased similarity observed. The influence of altered conditions from fresh to saltwater are however not the only variables impacting fish. Continued influence of external variables on fish throughout the sampling period seems apparent from results.

5.5.2.2 Localisation of microbial communities of gills

Use of biopsies allows appraisal of microbes present throughout the gill tissue, not merely the surface of gills. The community structure observed in the results of this research was therefore influenced by bacteria from all regions of the gills, including the mucus layer, lamellae, underside of the cartilaginous arch and other regions that cannot be reached by swabbing. Biopsy excision is also considered to sample microbes present within the tissue itself, below the tissue surface (Grice et al., 2008; Haalboom et al., 2018) Results therefore provide interesting insight then into the microbial community of the entire gill.

5.5.3 Influence of measured variables on gill microbial populations

Overall, the results support the theory of a gill-associated microbial community distinct from its environment (**Figure 5.5**), suggesting a generally similar microbiota. However, no single ASV (of 628 in total) was detected in all 124 biopsy samples, and only six ASVs were detected in >50% of total samples. So, although biopsies appear distinct

from environmental isolates, they do not appear at all homogenous. This is in contrast with previous studies, that have utilised parameters such as inclusion of only those taxa present within 90% of samples during the trial period (Lokesh and Kiron, 2015). This study differs from previous research in a number of ways, but most crucially appears to be the extended time course of analysis, and the variety of variables likely then imposed on fish.

As identified above through appraisal of **Figure 5.5**, a period of adaptation is apparent in the gill-associated microbial community following introduction of fish to the marine environment. Clear shifts in dominant phyla are also observable throughout the sampling period (**Figure 5.6**), with significant differences particularly in groups 1, group 4 and group 5. Group 1 might be explained by microbial adaptation to the marine environment, however variations in group 4 occurs following establishment of relative homeostasis. Variables other than adaptation to seawater must then be important in driving this divergence. Microbial community composition of surface epithelium in fish has been demonstrated to fluctuate with many factors, including stocking density, temperature, salinity and disease incidence (Fontaine et al., 2018; Ornelas-García et al., 2018; Sullam et al., 2009; Zha et al., 2018). Although fish were present within the confines of a net-pen, they were nonetheless exposed to all manner of environmental influences. The combined effect of these variables might go some way to explaining then the variation between sampled groups during the collection time-course.

5.5.3.1 Failure to identify a 'core' microbiome across sampling period

Previous research often promotes the existence of a 'core' microbial community within tissues, including the gills (Reverter et al., 2017), however such a shared population of bacteria was not particularly evident here. It seems likely that the extended nature of the sampling period of this research might explain this failure to identify shared microbiota. Fish were introduced to the marine environment shortly before commencement of sampling and increased in size and age throughout the collection period. Research in humans has demonstrated clear differences in microbial communities with age and diet of individuals (Yatsunenko et al., 2012), which if analogous to fish might explain the shifts observable in this salmonid dataset. Unlike short studies where the adherent

microbiota is affected by alteration of a single variable, this research demonstrates the continual modulation and adaptation of the gill microbiota. These shifts do not necessarily represent dysbiosis, but rather natural change and adaptation. The majority of studies describing core microbiota do so based on fish raised in a controlled environment or sampled at a small number of time-points. The results of this research suggest that what might be considered 'core' microbiota of Atlantic salmon might be more labile, changing with the altered intrinsic physiology of fish and influence of external environmental factors throughout their lifecycle to best facilitate function and survival.

5.5.3.2 Influence of external factors on microbial communities

Results of PERMANOVA and hierarchical cluster analysis indicate that the greatest influence on community composition is from external variables that impact the entire fish population. Despite apparent high individual variation between fish, samples cluster largely by sampling date (**Figure 5.8**). Sample group (likely impacted by fish age and prevailing environmental conditions) appears therefore the most significant factor impacting microbial diversity. Previous research demonstrates the significant restructuring of microbial communities with single variables, variables that likely impacted fish in combination and continually during the 12-month sampling period of this study. Based on the results of this research, on-farm events and pathology might also have initiated restructuring or dysbiosis in the adherent microbiota of these fish. A clear change was observed in microbial structure of gills after application of H_2O_2 treatment, for example, and results of PERMANOVA testing suggest an influence of gill disease, albeit not a significant one. Although there is little research on the impact of H_2O_2 on the microbiome, it is well understood that activities such as handling illicit stress and this can lead to impaired immunity with opportunity for microbial growth (Dhanasiri et al., 2011), with resulting altered microbial communities (Minniti et al., 2017). H_2O_2 is capable of killing many organisms in the marine environment, depending on concentration and sensitivity (Xenopoulos and Bird, 1997).

Environmental parameters collected routinely onsite by the producer SSF were unfortunately not made available to this research in time for analysis and writing, and so

their influence was not investigated. The influence of seasonal changes and production environment on the microbiome is however not a new concept (Giatsis et al., 2015; Gobet et al., 2018), with apparent greater influence of seasonality on fish skin populations than on internal gut microbiota (Arias et al., 2019).

External variables impact all fish within a group or a population. Variation between fish concurrently sampled within the same group (**Figure 5.7**) suggests as well the influence of individual factors, driving divergence between the microbial communities of specific fish. Previous research regarding fish reports too a high degree of community variation between individuals (Boutin et al., 2014; Fjellheim et al., 2012) but often does not suggest individual factors that might drive this variation. Research mainly in humans but also conducted in marine organisms has demonstrated alterations to the microbiome with incidence of diseases (Lloyd and Pespeni, 2018; Reid et al., 2017). These pathologies are not necessarily bacterial infections, rather an altered tissue state (and potential impairment of the immune system), which appears to result in significant, disadvantageous change to the microbial communities therein. The influence of disease was then considered a potential variable in driving individual variation in this study.

5.5.3.3 Pathology of as driver of individual variation

Histological assessment of gill tissue showed that severity of tissue damage varied between fish, even within concurrently obtained sample groups (**Figure 5.3**) and beta diversity through PCA did suggest an influence of gill pathology on community structure (**Figure 5.8**). Although gill pathology was not considered as important an explanatory variable in gill community variation as sample group (**Appendix C.5**), results suggest that pathology may drive subtle individual variation. The problem of collinearity was explored using RDA (Appendix C.5), however there appears little correlation of sample group and explanatory variables associated with gill disease. This was surprising, as severity of pathology was seen to alter seasonally in this dataset. This, along with limited numbers of fish observed to have severe pathology, suggested that collinearity might be an issue within the dataset. RDA analysis does not suggest a correlation of these variables (**Appendix C.5**), however this is a known incidental

(seasonal) association. Had analysis been performed across a shorter time period, or with sampling data as a factor rather than grouping group, a collinear relationship might have been observed (Dormann et al., 2013). Collinearity presents a problem of determining predictor variations for observed alterations, however two-way PERMANOVA results suggest a lack of significant interaction of these variables. The variation associated with gill pathology, although not significant at a community level, appears to due to specific, key taxa.

Of the key microbes considered to be associated with gill pathology within the results, some, but not all, are described as infective bacteria associated with gill disease in fish. The others might represent opportunistic or less competitive microbes, altered perhaps via loss of gill immune modulation, or with growth of competitive bacteria. This subtle restructuring of the transient microbial communities in more traumatised and thus functionally compromised gill tissue might then represent dysbiosis.

Significant differences in microbiota at various taxonomic levels with worsening fish pathology include altered relative abundance of Verrucomicrobia (**Figure 5.14**), with greater relative abundance in fish experiencing severe histological change. The relative ratio of this phylum to Bacteroidetes varies significantly between healthy and diseased fish. Relative ratios are utilised in human medicine as indicators of community restructuring in the microbiota of tissues such as gut and skin (Bervoets et al., 2013; Williams et al., 2019). These ratios are applied as prognostic indicators as well as indicators of resolution or worsening of various disease states. At the genus level, *Candidatus Branchiomonas* and *Candidatus Fritschea* were observed in significantly different levels with different pathology classifications. These microbes have been previously implicated as microbial pathogens in the salmon disease epitheliocystis. Greater apparent abundance of these taxa is therefore not entirely surprising in the context of worsened gill disease, although epitheliocystis was not observed in the majority of gill sections from which they were isolated. It is possible then that fish might have been experiencing early or mild infection with epitheliocystis, although *Candidatus Branchiomonas* has been previously isolated as an abundant component of the rainbow trout gill microbiome without any apparent ill-effect in host fish (Brown et

al., 2019; Downes et al., 2018; Gunnarsson et al., 2017). Results might then indicate that instead of being the sole infectious agent of epitheliocystis, *Candidatus Branchiomonas* is a resident of the salmonid microbiome, the relative abundance of which is altered during this gill pathology. This would certainly assist in explaining the reported phenomenon of so many causative agents of this disorder. The additional bacterial genus identified as present in significantly greater abundance in diseased gills, *Rubritalea*, is not considered a bacterial pathogen. Together these microbes might therefore be components of the microbiome, the abundance of which is altered with disease, and useful as indicators of dysbiosis or early indicators of microscopic gill damage.

The abundant microbe identified as *Procabacteriaceae* was found to be of lower abundance in gill tissue with greatest observable pathology. This is a bacterium for which little information is available, however, it is known to be an obligate symbiont of acanthamoeba The infectious amoebic condition AGD is of particular concern to aquaculture (Crosbie et al., 2012; Haugland et al., 2017), and although research indicates that a number of amoebae might be associated with this disease (English et al., 2019) the apparent common causative agent is *Neoparamoeba perurans* (Crosbie et al., 2012). Acanthamoeba are distantly related to paramoeba, identified as occasional infectious agents of fish (Dykova et al., 1999) although also noted without apparent disease on the gills of Atlantic salmon (Howard, 2001). In this case, if indeed *Procabacteriaceae* is again an obligate endosymbiont sampled through biopsy excision of gill tissue, its presence too appears benign, and potentially a reasonable indicator of gill health in this population of farmed fish.

5.5.3.4 Additional factors influencing the microbial communities of gills

In addition to the apparent influence of gill pathology, a number of other variables are highlighted by this research as influencing the microbial community of Atlantic salmon gills. Of particular note are the findings regarding acclimatisation to seawater, and the treatment with H_2O_2. Alterations with salinity are noted in previous research (Lokesh and Kiron, 2015). Those authors noted the increased abundance of Proteobacteria with

duration in the marine environment, observed in this research too. Unlike their work however, this study found Chlamydia abundance as also significantly altered during adjustment to saltwater of sampled fish. Many factors might account for this difference in results, including use of sampling technique and different fish populations. Fish housed in laboratory conditions have been demonstrated to encounter and acquire different environmental microbial communities (Webster et al., 2018), influenced too by the impacts of handling, diet and genetics. These results support the conclusion that laboratory-raised fish are a poor proxy for the study of the microbial community of marine-raised farmed fish. Although this was a responsible sampling regime, and samples were not collected as regular intervals, these results do represent the 'true' experience of farmed fish. This study represents the first investigation of microbial community change with application of H_2O_2. Many of the microbes in pre-treatment samples were no longer identified in post-H_2O_2 treatment fish (**Table 5.2**). The majority of previously abundant taxa, including *Procabacteriaceae*, *Pseudoalteromonas* and *Psychrobacte*r, decreased in relative abundance, suggesting a negative impact of H_2O_2 on these microbes.. No significant reduction was observed in disease associated *C. Branchiomonas* however, and reportedly pathogenic *C. Piscichlamydia* is even observed to significantly increase in relative abundance, suggesting these microbes are not sensitive to H_2O_2 treatment. These results suggest that benign or even beneficial components of the microbial community can be cleared by H_2O_2 treatment., followed by recolonization with a mixed, low-abundance community likely acquired from the environmental bacteria.

This research therefore has implications for production of Atlantic salmon. Their treatment with H_2O_2 to control sea lice and AGD might be associated with removal of harmless adherent microbes. If *Procabacteriaceae* is indeed an indicator of the presence of a harmless acanthamoeba, it appears H_2O_2 clears this protozoan along with its harmful amoeba it targets (Adams et al., 2012). Despite the reportedly effective treatment of AGD, H_2O_2 can also be harmful (Avendaño-Herrera et al., 2006b; Kiemer and Black, 1997). Fish deaths following AGD treatment are well documented, currently attributed to gill compromise and the stress of treatment leading to mortalities. Future research then also consider the restructuring of microbial communities that occurs with

H_2O_2 application too. No investigation of the functional activity of microbes was performed. Had time allowed, results of this might have been informative regarding biological activity of various microbes, particularly those identified as associated with different disease states.

5.6 Conclusion

This dataset represents the first study of both the histopathology and gill microbiome of Atlantic salmon through nearly an entire production cycle. Fish were sampled for 12 months from soon after their entry to seawater, allowing analysis of almost the entire marine-phase of Atlantic salmon production. In addition to this, results include interesting findings regarding the factors influencing the microbiota during this time.

That sampling date was the most significant driver of variation between fish, with continual restructuring of gill microbial communities raising questions about the previously assumed stability of microbial communities of gills, and current thinking regarding the 'core' microbiota. The microbial community of younger fish at the initiation of the sampling period was identified as distinct from subsequent samples, likely due to adaptation to the marine environment. Even following this adaption however, the microbial community did not remain static. The multitude of factors in the marine environment and on-farm events appear to result in a constant fluctuation in the microbial communities of the farmed fish. Whilst this was not unexpected, these results appear to suggest no constant core community on gills over the 12-month sampling period. These results indicate instead that, rather than a consistent community maintained throughout the marine stage, microbial populations of gills are constantly restructuring, with few shared taxa across the sampling period. Perhaps the microbiome of the GIT requires a stable ‘core’ community, for digestive function, but gill populations are more labile. The resident bacteria of gills were influenced by a number of identified factors during the study period, including on-farm application of H_2O_2, suggesting that the current method of farming Atlantic salmon in Scotland might partially be responsible for the changing microbiota. Whether through adaptation or dysbiosis, perhaps this goes some way to explaining, in conjunction with previously described reduced genetic diversity and rearing environment, the divergence in many characteristics of farmed fish relative to their wild counterparts, including susceptibility to disease.

One clear modulator of the adherent microbial communities was application of H_2O_2. Abundance of many bacteria were altered by this chemical treatment and, crucially, bacteria previously identified as present in the majority of individuals within the

population were cleared. It appears, therefore, that application of H_2O_2 removes previously common microbiota that might have been considered a part of the healthy community. Following H_2O_2 application, individual variation appears to increase between fish, with recolonization of gills with different low abundance environmental bacteria. These results have clear implications for the use of H_2O_2 in the production of salmonids, where this chemical is frequently used to treat fish, from eggs to adults. Application of H_2O_2 is known to be dangerous to fish already suffering from gill disease, presumably due to overload of already compromised immune and functional capacities. These results suggest its application might also negatively impact the gill microbial communities. Clearance of many microbes from gills by H_2O_2, but failure to reduce relative abundance of those bacteria typically associated with disease, like *Tenacibaculum* and *C. Branchiomonas*, might give these pathogens a competitive advantage in colonisation of tissue.

As expected, fish in this study seemed to have encountered a number of infectious challenges during the study period. Characteristic AGD lesions were seen in many of the fish through gross assessment, and amoeba were observed in histological sections. Despite few observations of histological lesions indicative of epitheliocystis, it is possible that this infection was present within fish too, based on isolation of *C. Branchiomonas* and *C. Piscichlamydia*. Due to the complex nature of gill disease and the concurrently identified pathologies, results of this research cannot be related to a single infectious agent. Instead, through assessment of histology, a general picture of gill health was obtained, rather than specific disease diagnoses. This was related to the adherent microbial communities in an effort to understand the microbiota of both healthy and damaged gills. Through this, a small number of microbes were identified as potential candidate biomarkers of health in gills. *C. Branchiomonas* and *Rubritalea* are suggested as potential microbial biomarkers of gill pathology. Relative ratio of Verrucomicrobia: Bacteroidetes too appears useful in the prediction of the degree of tissue pathology. *Procabacteriaceae* on the other hand appears an indicator of the healthy microbiome.

Overall, because of the interplay of multiple environmental stressors and potentially even unaccounted variables, results presented an analytical challenge. They do, however, provide interesting preliminary insight into factors that might merit further research as to their impact on microbial community structure of the gills of farmed salmon. This work therefore presents a number of interesting questions regarding the function and modulation of the gill adherent microbial community in farmed Atlantic salmon. Dysbiosis is broadly defined as the disarrangement of the normal microbiota with negative host consequence, and an argument could be made that dysbiosis is occurring within this dataset. By collection of fish from the marine environment instead of observation in a controlled trial, these results present a more realistic view of the microbiota of farmed salmon. It may be that there is no 'normal' or 'healthy' microbiome within these fish, and this research identifies potential markers of monitoring optional or dysbiotic change.

6. Utility of swabs versus biopsy collection in assessment of the microbiome of gills

6.1 Overview

Through sample collection as part of Chapter 5 of this thesis, tissue biopsies of Atlantic salmon gill tissue were obtained and fixed, before extraction of DNA and sequencing of microbial communities. Biopsies were selected as the sampling method of choice based on the large surface area of gill tissue and potential for microbiota to be located not only on the tissue surface within easily accessible mucus layer, but also more cryptically in folds and small tissue cavities that might not be accessed by swabbing. Human dermal layers have been demonstrated to host significantly different microbial communities to that of the skin surface, meaning they cannot be sampled by swabbing alone. It was therefore determined that, in addition to assessment of the biopsies utilised in Chapter 5, a comparison would be made to swab-derived isolates obtained from the contralateral gill of the same fish. Results of this comparison are presented in this chapter, and they demonstrate a clear difference in community composition with sampling methodology employed. Sampling method is observed to have an influence on obtained microbial populations, with significant differences identified at various levels of taxonomic classification. Overall, biopsies appear to have a utility in isolation of a limited number of taxa not identified by swabbing, although suffer from high host DNA inclusion. Swabbing on the other hand results in a greater number of sequencing reads, likely due to lower host contamination, and isolates a more diverse microbial community, however more closely mimics environmental isolates. Whether this is due to true greater similarly or greater unintended inclusion of environmental microbes through selective collection from the gill surface by swabbing cannot be confirmed. However, what is clear is that sampling methodology significantly influences results, and that there exist different indications for different sampling methodologies with varied experimental aims.

6.2 Introduction

With recent advances in metagenomic technologies available to researchers, use of techniques such as next generation sequencing in exploration of microbial community composition has become common, particularly in the use of the conserved microbial 16S genomic region. Use of 16S has allowed exploration of bacterial communities from all areas of the planet – from deep ocean sediment, to gastrointestinal communities of alpine parrots. Insight into the microbial communities enhances our knowledge of the importance of these microbes, their relationships and functions in overall ecology. However, many factors impact the results of studies in microbial community composition. Bias can be introduced by a variety of means, for example, by PCR amplification, due to the variable numbers of copies of the 16S gene in different genera. Another common set of problems is issues such as handling and contamination, which need to be controlled for as much as possible through use of controls and other checks. If representative samples are not obtained in the first place, any results might be incomplete or misleading. The focus of this chapter is in exploration of whether the method of obtaining the samples provides suitably representative samples in the first place, and how results can be influenced by sampling methodology.

6.2.1 Impact of study design on microbial investigations

A great deal of literature is available regarding the storage and transportation of microbial samples, including media for fixation and temperatures for transport and long-term storage (Bai et al., 2012; Hildonen et al., 2019; Hill et al., 2016). Limited information, however, is available regarding the impact of actual sampling method on collection. Previous research demonstrates a significant impact on assessments of community structure with different methods of fixation and extraction of samples. Due to the potential for rapid growth of contaminant bacteria as well as alternation of resident microbial ratios through growth or degradation, microbial samples cannot be transported ‘as-is’. Sampling regimes must be designed to conserve baseline microbial abundances and prevent alterations to community composition that will impact eventual results (Hale et al., 2016; Vandeputte et al., 2017). The gold standard of microbial

preservation is to freeze samples immediately, with rapid reduction in temperature recommended by many publications. (Hale et al., 2016; Hildonen et al., 2019; Song et al., 2016a). Storage below -20 C is thought to most adequately preserve microbial communities (Bai et al., 2012), and as different storage methods have been demonstrated to significantly impact eventual results (Choo et al., 2015), storage is certainly an important consideration. Unfortunately though, it is not always practical or even possible to freeze samples in a field situation. Instead, various methods of preservation have been trialled, with varied success (Osman et al., 2018; Song et al., 2016b). Many of the methods of traditional DNA fixation are not appropriate for preserving microbial DNA, and conflicting information is available regarding the efficiency of fixatives such as ethanol (Hildonen et al., 2019; Spigelman et al., 2001) and RNAlater (Drew et al., 2016; Franzosa et al., 2014; Sinha et al., 2016). Method of DNA extraction has also been demonstrated to greatly alter obtained results, with different results obtained using different kits or protocols (Henderson et al., 2013; Luo et al., 2007; Salter et al., 2014)

Contamination is of course also a consideration, from initial sample collection all the way to final sequencing (Weiss et al., 2014). Well recognized contaminant microbes of kits include *Rhodococcus* and *Acinetobacter*, to name just two (Mohammadi et al., 2005; Salter et al., 2014; Stinson et al., 2019). Contamination of samples with environmental or human DNA will negatively impact results too (Glassing et al., 2016; J. Kumar et al., 2016; Laurence et al., 2014; Sehn et al., 2015; Weiss et al., 2014).

Although there appears to be a large volume of research on optimizing protocols and maintaining reproducibility in metagenomic studies of the microbiome, much of it is in terrestrial organisms, and the majority focus on the study of the gut microbiota. Much work is therefore still to be conducted in the optimization of study design for the research of aquatic organisms such as teleost fish. The studies that do exist in this field largely mirror the results of human research, demonstrating an impact of sample storage and extraction in eventual results (Hildonen et al., 2019; Larsen et al., 2014).

A topic that has not to our knowledge been addressed by existing research is the impact of actual sampling methodology on results of community composition in the study of the fish microbiome. A small number of studies have sought to address this question in the research of human microbial communities of gut and skin, with their results suggesting the method of sample collection does have a significant impact on obtained microbiota (Prast-Nielsen et al., 2019). The majority of previous research on the adherent microbial populations of salmonids obtained samples via swabbing.

6.2.2 Varied methodology

Tissue origin and sampling methodology is thought to have a significant impact on microbial community analyses. Historically, identification of microorganisms such as bacteria could only be performed through culture, a technique that suffers from a high incidence of errors in colony identification, as well as limited scope in accurate taxonomic description of novel species. It is also well-known that culturing fails to capture the full microbial diversity of bacterial populations present, regardless of sample origin (Hiergeist et al., 2015; Joint et al., 2010). Modern molecular methods, such as the metagenomic technique of next-generation sequencing of the 16S gene allows for analysis of a much broader and representative diversity of microbial populations in samples (Salipante et al., 2013). This is often also of important clinical relevance (Cao et al., 2017; Didelot et al., 2016). These techniques do however also have associated biases, such as variable success of PCR for microbes with varied copy numbers of the 16S gene (Aird et al., 2011; Kebschull and Zador, 2015; Kennedy et al., 2014; Sze et al., 2019), and variable results depending on the region of the 16S gene sequenced (Pollock et al., 2018; Yarza et al., 2014). However, the approach is still considered superior in capturing the microbial diversity.

Minimising error and bias in these techniques should be considered a priority in microbial research, to ensure accurate estimation of community composition. A variety of methods have been employed previously in investigation of the microbial communities of fish tissue, and a number of studies exist on validation of storage techniques as well as methods for DNA extraction. Many studies contrast the microbial

communities of different tissue sites in fish (Brown et al., 2019; Cahill, 1990), but none to our knowledge consider the impact of sample collection on the eventual results.

Populations of microbes are known to differ between tissue site (Legrand et al., 2018), and even between discrete regions of the same tissue. For example, microbial communities within different regions of the respiratory (Glendinning et al., 2016) and GIT (Lavelle et al., 2015) have been observed to vary significantly. Even human conjunctival microbial communities have been demonstrated as significantly different from limbus and fornix tissue, all within the eye (Ozkan et al., 2018). It appears therefore that samples must be obtained carefully, with accurate distinction in tissue site of origin and in aseptic technique (Weiss et al., 2014). For studies interested in the composition of bacterial populations of the GIT, samples can be obtained by biopsy of the gut of living or dead tissue specimens (Huse et al., 2014; Lavelle et al., 2015; Osman et al., 2018), as well as through swabbing (Budding et al., 2014; Vandeputte et al., 2017) or analysis of faecal matter (Amato et al., 2013; Clayton et al., 2016). Faecal sampling, or otherwise obtaining microbial samples from communities considered 'representative' rather than directly from the tissue of interest, raises a number of problems in sample collection. Anatomical and physiological factors can result in samples not being representative of the community of the organ of origin. For example, in faecal sampling, the digestion process can alter microbial populations so as to remove important genera from the final sample. Foregut fermenters utilize important foregut microbes in digestion that are removed from faeces through subsequent glandular digestions prior to defaecation (Davies et al., 1994). Investigation of rumen microbial contents must be performed through oral stomach tubing or rumen fistula (Henderson et al., 2013; Martinez-Fernandez et al., 2019), with results obtained from faeces not only unrepresentative of the rumen communities, but also less diverse and with less individual variation (Mu et al., 2019). Sampling directly from the site of interest therefore appears desirable for investigating microbial community composition.

6.2.2.1 Biopsies

Tissue biopsies are useful for analysis of the adherent microbial community or even intracellular symbionts through direct sampling from the area of interest. Biopsies have been used in a variety of studies, from investigation of the skin microbiota or microbial communities associated with cancer in humans (Grice et al., 2008; Meng et al., 2018) to investigating temporal differences in the microbiomes of humpback whales and profiling the progression of disease in starfish (Bierlich et al., 2018; Lloyd and Pespeni, 2018) . Live biopsies can be obtained through excision of small tissue sections (Grice et al., 2008)or needle aspiration (Meng et al., 2018). These have the advantage of utility in humans and other species without the need for euthanasia of individuals before-hand, as well as allowing for repeated sampling of the same individual. They do however involve complications, such as limited tissue coverage and negative impacts on the sampled individual such as bleeding (Huse et al., 2014). They also present the issue of a high level of contamination with host cells. Nevertheless, live biopsies can be useful in determining the impact of treatments (Doherty et al., 2018) or the progression of disease (Lloyd and Pespeni, 2018; Meng et al., 2018), although there is the potential for disruption of microbial communities through trauma or bacterial introduction with this methodology.

6.2.2.2 Swabbing

Aseptic or sterile technique is essential for sample collection in any form, to prevent contamination of samples. Fieldwork presents a challenge in this context, as sterile conditions can be problematic to achieve and maintain (Merrifield and Rodiles, 2015). Many studies utilize swabbing for sample collection due to its ease, particularly in fieldwork situations or contaminated environments. Application of a swab is simple and requires less experience and aseptic technique than excision of a tissue biopsy (Ratliff, 2001; Tedeschi et al., 2017). Swabbing also allows repeated sampling of a live individual without the level of tissue damage associated with biopsies, although swabbing is not entirely benign and does present the potential for mild tissue trauma in delicate tissues or wounds, with associated alterations to microbial community composition from repeated swabbing. One of the more important benefits of swabs,

however, is the ability to obtain samples without a large amount of associated host DNA. Little research exists in this area, however intuitively one would suppose a larger proportional inclusion of host cells through biopsy excision.

When swab collection is contrasted to tape-strip collection in investigation of the human epidermal microbiome, results are comparable (Ogai et al., 2018). However, when the sampling methodologies of swabbing and excision through biopsy were contrasted in investigation of the microbiota of human skin (Prast-Nielsen et al., 2019), respiratory (Glendinning et al., 2017) and rectal tissue (Araújo-pérez et al., 2012), significant differences in microbial community composition, richness and diversity were observed. This is potentially due to the presence of microbiota in areas that cannot be accessed by swabbing, such as the dermis, which means swabbing appears less efficient in isolation of pathogenic microbes of diseases that originate in this location, such as psoriasis in humans. Biopsies are documented as indicated in the assessment of deep tissue infections where swabbing has proven inefficient in detection of clinically relevant microbes (Tedeschi et al., 2017). Other studies, however, concluded that the methodologies are interchangeable with comparable success in microbial isolation (Haalboom et al., 2018; Hanshew et al., 2017; Rondas et al., 2013). If differences in community composition are detectable between swab and biopsy-derived samples in other species and other tissues, sampling methodology might have a serious impact on results in the context of studying the salmonid microbiome.

6.2.3 Microbial research in fish

The composition of microbial communities and their activity as part of the microbiome of fish is a fascinating and expanding field of research. Whilst the majority of studies have focused on the bacteria present within the digestive tract, research has also been conducted on the microbes of other epithelial surfaces, such as the skin and gills. Interactions of the fish host and resident bacteria has been suggested to have a role in the action of digestion (Dehler et al., 2017a; Egerton et al., 2018; Llewellyn et al., 2016), adaptation to environmental conditions (Webster et al., 2018) and even in immune function (Brown et al., 2019; Liu et al., 2014).

Previous research investigating the bacterial communities of salmonids, including rainbow trout as well as Atlantic and Pacific salmon, have produced many interesting results. They have identified potential microbial biomarkers of disease, microbes suggested to be associated with disease resistance (Araújo et al., 2015; Brown et al., 2019; Legrand et al., 2018; Llewellyn et al., 2014) and even divergent ratios of microbial community composition in different disease states, where taxa present within the community are largely unaltered, but significant differences in proportional representation are associated with susceptibility to and incidence of disease in fish (Legrand et al., 2018; Sandve et al., 2017). Although this remains an early field of research, results provide exciting insight into the possibly roles of microbiota in fish health. The majority of previous studies into the adherent microbial communities in fish utilized swabbing for sample collection. This method is certainly user friendly, as swabbing is simple and convenient to perform, with the potential for samples to be frozen or combined with fixative solution for transport and storage. It is for these reasons, along with the non-destructive nature of collection, that likely makes swabbing the commonly performed method of sample collection in studies of the microbiota of fish.

Similar to the results of human research, studies investigating the microbiota of fish have observed distinct microbial communities with different tissue localities. Bacterial populations of the GIT vary with biogeography, influenced it is thought by variable oxygen, antimicrobial peptides and pH variability (Donaldson et al., 2017), with further niche partitioning noted in specific tissues. Previous research has recorded the presence of distinct microbial layers within gut biofilms, with preference of different taxa for closer or more distant association with the luminal wall (Palestrant et al., 2004). Spatial organisation of microbiota therefore appears complex, with intra- and inter-species interactions occurring on top of host-driven modulation (Liu et al., 2016; Tropini et al., 2017). This level of investigation has not yet been applied to the microbial communities of gill tissue, however previous research suggested that gill-specific microbial communities might only be observed in more cryptic locations of gills, such as between pharyngeal arches and lamellae, due to the continuous environmental exposure of other

gill surfaces and constant water flow (Mudarris and Austin, 1988). Whilst we now know that the adherent microbiota of gills is specialized and distinct from environmental communities (Lowrey et al., 2015; Pratte et al., 2018), there may exist as yet unexplored niche partitioning of microbiota within tissue, driven by anatomical and physiological differences in localization, such as within mucoid covering or between lamellae.

6.2.4 Optimal methodology in exploration of the salmonid microbiome

If alternative methodology in sample collection does indeed impact results, this has serious implications for the field of microbial research. Whilst results obtained in a single study through identical methodology will be comparable, results across studies may not. Results of swab and biopsy comparison in human research suggest differences may be observed, depending on study aim and the tissue of interest (Ratliff, 2001; Tedeschi et al., 2017). Sample storage and extraction are already documented as significantly impacting community composition. This chapter therefore sought to answer the question, does sampling methodology impact results when investigating the adherent microbial populations of salmonid gill tissue? A number of studies in human medicine have asked a similar question (Ratliff, 2001; Tedeschi et al., 2017)., and some have obtained results that suggest sampling methodology does significantly impact surveys of microbial communities (Prast-Nielsen et al., 2019; Zmora et al., 2018). This work was conducted using a subset of biopsy-derived sequencing results obtained as part of work for Chapter 5, with additional sequencing of samples obtained by swabbing. Through use of swab and tissue biopsy-derived DNA samples, results were contrasted to investigate the impact of sample type (and therefore too, sampling location) on microbial richness, diversity and relative community composition. Clear differences were observed between samples obtained by swabbing versus biopsy excision.

6.3 Methods

6.3.1 Sample collection

For exploration of the Atlantic salmon microbiome, swabs and biopsy samples were obtained and sequenced from a total of ten individuals. Swabs were collected by use of a sterile cotton swab applied to an equivalent area of gill surface excised for biopsy fixation. Instead of excision of tissue on right side however, a swab was passed progressively over the entire identified region of tissue and placed in appropriate fixative. Additional details of biopsy sample collection are provided in Methods (Section 2.2.1). Swab specifically were obtained from the first gill arch, contralateral to the location of biopsy collection. Although previous literature has included a wash step for removal of non-adherent environmental microbes prior to sample collection, this was not performed. Following application of swabs to the right-side first gill arch across an area equivalent to that excised and fixed as part of biopsy collection, swabs were also fixed in RNAlater. Swabs were collected from additional fish and ethanol fixed using the same methodology. Other than the method of collection, and unless otherwise stated, samples were treated identically following this step.

DNA was extracted from samples fixed in RNAlater. Full details of this protocol are provided in Methods (Section 2.3.2) Briefly, swabs were dried and extraction performed identically to biopsy-derived samples, with use of phenol/chloroform and then using the DNeasy Blood + Tissue extraction kit (Qiagen), following the manufacturer's recommendations. Sample aliquots for DNA extraction were only removed from freezers a maximum of two times, to minimize any freeze-thaw impact on DNA integrity (Grecz et al., 1980; Ross et al., 1990; Singh and Baghela, 2017) and community abundance (Song et al., 2016a). Each swab was used in its entirety and although DNA extractions could be archived for future use, original swab samples could not.

DNA extraction yields from swabs were similar to concentrations obtained from gill biopsies (50 – 100 ng/ml), however, unlike biopsy-derived samples, pooling of

biological replicate swab extractions was not performed. Duplicate extractions were obtained from the same swab not to increase sampled area, but to maximise DNA yield from a single sample. This was considered appropriate as swabs were applied to a larger area of gill surface tissue than could be extracted as part of biopsy analysis, similar to the entire fixed area for biopsies. Multiple extractions were performed from the smaller sections removed from this fixed biopsy tissue to maximise the gill area assessed, however, repeated swabbing over an identical area would have represented unnecessary technical replication, and therefore not performed.

6.3.2 Next generation sequencing

Library generation and preparation for next generation sequencing of the V3/V4 region of 16S microbial genomic material was performed as described in Methods (Section 2.6). Initial results of PCR amplification using 16S primers of swab-derived DNA contained a notably greater concentration of PCR-product after clean-up (average 8.2 ng/µl) relative to individual biopsy samples (1 ng/µl). A total of ten swab-derived samples were sequenced in an identical methodology to tissue biopsy samples. Of these ten samples, one was subsequently excluded from analysis due to failure of the sequencing of the corresponding gill biopsy, and so lack of a comparison sample. Swab derived results for comparison to biopsy samples were therefore obtained for a total of 9 fish, from sampling Groups 8 and 9.

6.3.3 Computational analysis

Initial results of sequencing were analysed identically to biopsy samples using the latest version of Quantitative Insights Into Microbial Ecology 2, Qiime2 v2019.1 (Bolyen et al., 2019; Caporaso et al., 2012) (full details in Section 2.6.5). Demultiplexed pair-end sequence reads were pre-processed using DADA2 for quality filtering, denoising, joining of paired-end reads and removal of chimeras. The dataset was rarefied to a sampling depth of 1200 using Qiime2 for use in alpha diversity metrics. ASVs were identified using the SILVA 128 reference database (13.8 version) (Quast et al., 2013). Filtering to remove all non-microbial sequences was then performed.

Abundance profiles were calculated based on total read counts and multivariate analysis performed using Bray-Curtis resemblance matrixes in the ecological modelling program Primer version 7 unless otherwise stated. For statistical analyses, fish were split into groups according to sampling methodology, as well as by sampling date, and histological score assessment (None, Mild, Moderate or Severe) as described in Chapter 5. Alpha diversity indices were obtained from both rarefied processed reads in Primer 7. Figures were generated using Primer 2 as well as Vegan and Bioconductor packages in R.

6.4 Results

As an adjunct to exploration of the bacterial community composition of the gills in Chapter 5, swab samples were obtained from contralateral gill tissue in a number of individual fish. DNA was fixed, extracted and sequenced in an identical fashion to the biopsy samples described in chapter 5, to obtain duplicate isolates of the microbial community from fish; one from swabbing and another biopsy-derived. These isolates were utilised to compare the sampling methodologies of swabbing and biopsy excision in exploration of the adherent microbial community of gill tissue. Nine Atlantic salmon individuals were assessed, with additional sequencing of water and PCR controls. From these, a total of 119,258 pre-processed reads were obtained from swabs, with an average of 11,435 quality-filtered reads per sample after processing. Corresponding biopsy results were for a total of 80,111 pre-processed reads and an average of 2,352 quality-filtered reads per sample after processing.

A total of 303 ASV's were identified from sequencing the swab-derived samples. This equated to 260 genera, 164 families, 99 orders, 51 classes and 24 phyla. Similar to gill biopsy samples, a number of the identified phyla were of taxonomically ambiguous classification. Total ASV's obtained in corresponding biopsy-derived samples from the same individuals are lower, numbering 131, representing 114 genera, 86 families, 57 orders, 33 classes and 14 phyla.

6.4.1 Overall community composition varies with sampling methodology

6.4.1.1 Clustering of isolates by sampling methodology

Ordination of samples was initially performed using non-metric Multi-Dimensional Scaling (nMDS) using Bray-Curtis indices for observation of the relationship of individual isolates (**Figure 6.1**). Clustering of samples obtained by swabbing, biopsies and from environmental controls clearly distinguishes groups by sampling methodology. Plotted variables demonstrate no clear association of samples obtained from the same individual. Rather, swab-derived samples are seen in close association,

separate from the plotted values for biopsy-derived samples. Clustering was evident for samples obtained on both dates, from Group 8 and Group 9. Interestingly, swabs appear in closer proximity to environmental microbial communities than biopsy-derived samples. This may be due to a proportionally greater contamination of swab-derived samples with environmental bacteria, or indicate a less specialised microbial community of more accessible gill tissue, reflecting differences in microbial community composition and function in discrete gill regions.

Group average hierarchical clustering of 16S sequencing results from swabs and biopsies was also performed using Bray-Curtis similarities (**Figure 6.2**). The Bray-Curtis similarity matrix generated for swabs and biopsies showed marked differences between samples from the same individual, with results suggesting greater similarity between identically obtained samples from different fish than samples obtained by different methodology from the same individual. Hierarchical clustering of samples demonstrated two main clusters, labelled Cluster 1 and Cluster 2 (**Figure 6.2**). Cluster 1 is composed mainly of swab-derived isolates (90% swabs, 10% biopsies), and Cluster 2 is composed entirely of gill biopsy samples (100%). Disease status had no apparent impact on clustering results (**Appendix D.1**). Clustering of samples largely by sampling methodology suggests a difference in bacterial community coverage obtained with biopsy versus swab-derived samples, independent of disease status and time of sample collection.

6.4.1.2 Alpha Diversity

In assessment of the alpha diversity within communities, ASV richness, Shannon and Simpson diversity indices were calculated (**Figure 6.3).** These measurements were performed on a dataset rarefied to a sequencing depth of 1200 sequences, due to the unequal sampling intensity resulting from higher sequencing yield from swab isolates, to prevent bias of richness estimates (**Figure 6.3**). Community richness and diversity is clearly greater in swab-derived communities, initially suspected to be due to a vastly greater sequencing depth of microbial material from these samples, however following rarefaction, results of diversity analysis still indicate that swabbing isolates a

significantly richer, more diverse microbial community than biopsy collection. Results therefore indicate significant differences between the results of swabbing and biopsy collection, and that despite samples being obtained from the same original community, swab collection obtains a universally more diverse and microbially rich dataset.

Figure 6.1 nMDS of community origin

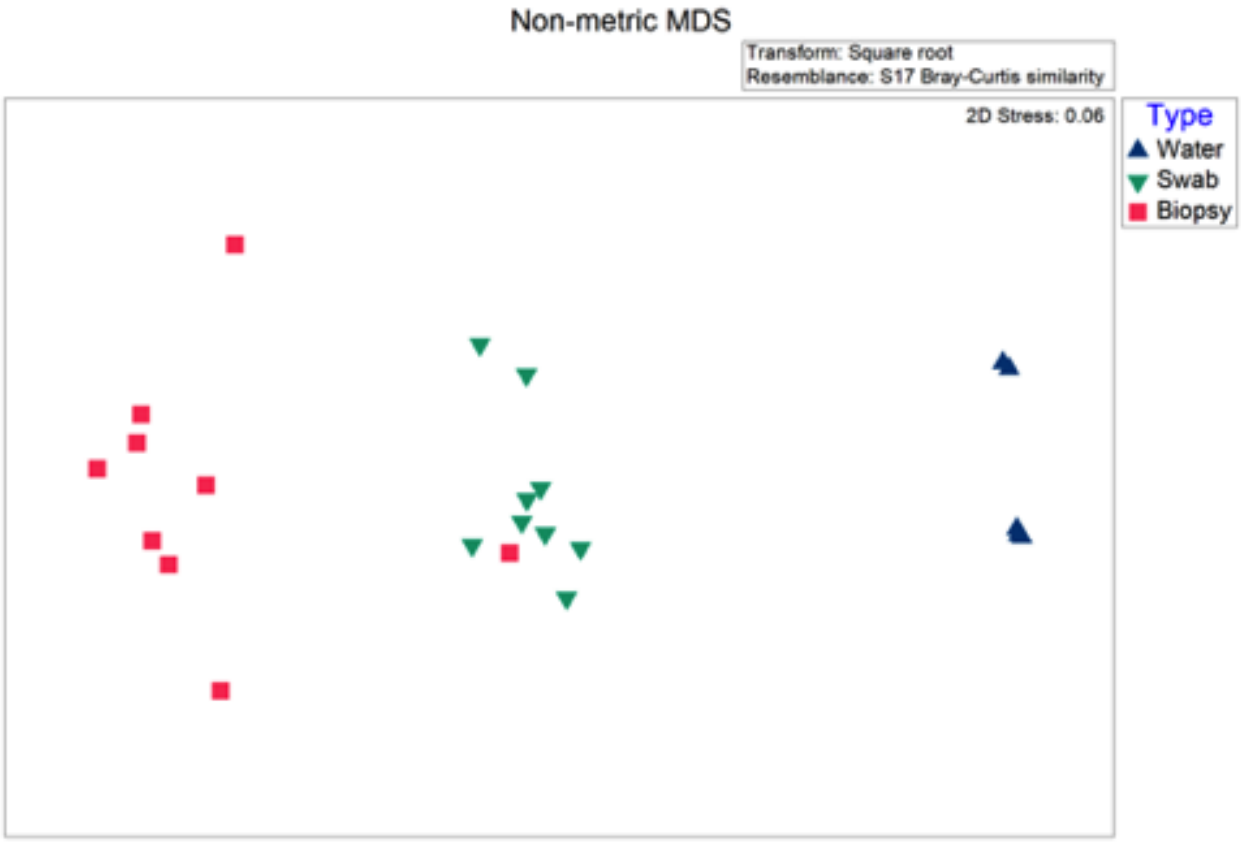


Figure 6.1*: non-metric multidimensional scaling plot of sequencing results (at ASV level) of swab, biopsy and water samples. Plot was constructed using Bray-Curtis similarity matrix generated from 16S sequencing results of 9 fish, performed in duplicate from swab (green) and biopsy (red) samples at ASV level. Environmental controls (blue) were obtained from water samples.*

Figure 6.2 Cluster analysis swab and biopsy communities

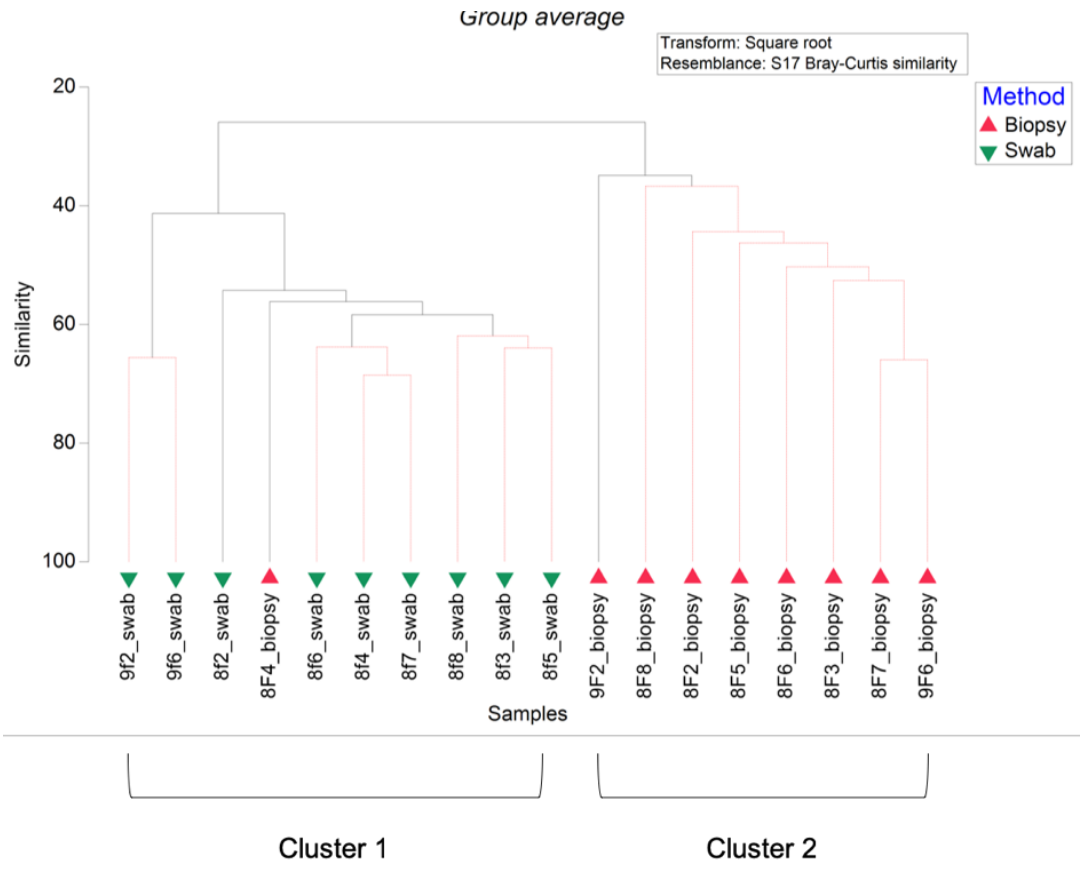
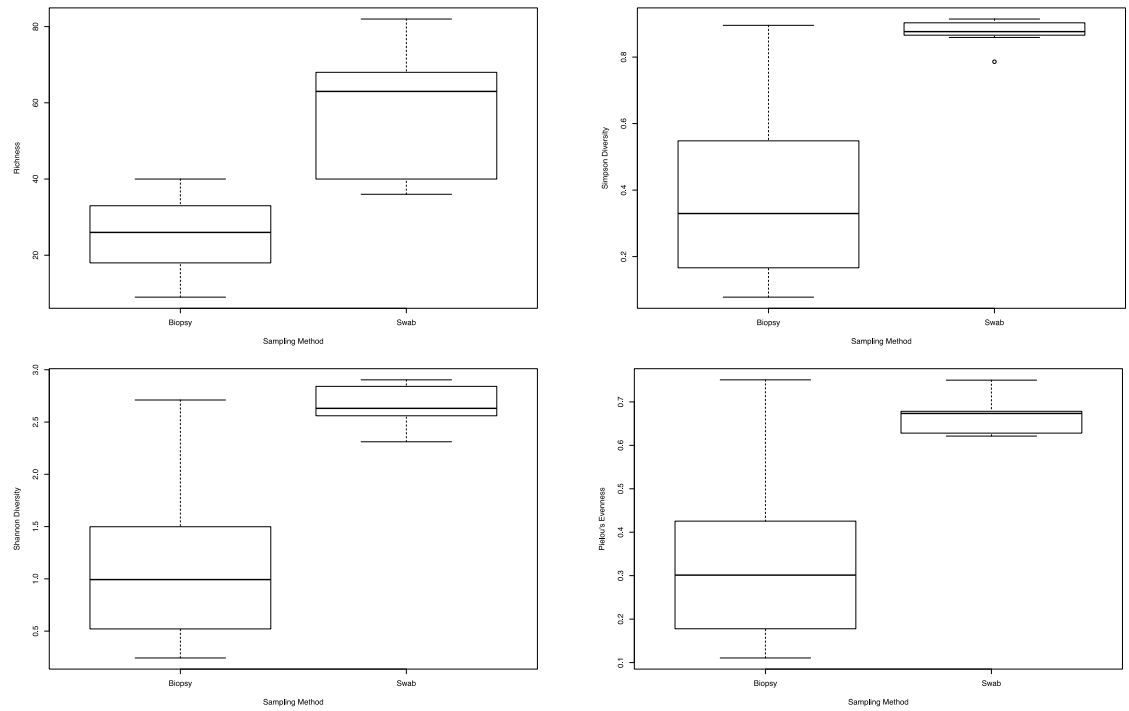


Figure 6.2: *Hierarchical cluster analysis of swab and biopsy samples. Group average cluster analysis was performed using Bray-Curtis similarity measures of square-root transformed ASV level datasets. Rather than association by group of sampling, as might be expected for samples obtained from the same individual, samples clearly orientate into two clusters, dominated by swabs, or biopsy derived results.*

Figure 6.3 Diversity plots

A B



C D

Figure 6.3: *Richness (A), Simpson (B), Shannon (C) and Pielou's Evenness (D) plots illustrate the clear differences in alpha diversity obtained from swab and biopsy samples. Indices were calculated as described in the methods on a dataset rarefied to 1200 sequences using Primer 7.*

6.4.1.3 Beta Diversity

Additional multivariate analysis was performed to investigate diversity between microbial communities including PCoA and PCA (not shown). In both multivariate analyses employed, samples appear to separate by sampling methodology along the axis of greatest variation and by sampling date in the second dimension. No apparent relationship between concurrently obtained samples is observable. Factors with greatest contribution to PC1 included *Procabacteriaceae* and *Candidatus Branchiomonas* (microbes identified with greater average abundance in biopsy-derived samples), as well

as *Psychrobacter* and unknown *Flavobacteriaceae* (identified with greater average abundance in swab-derived samples). Investigation of other microbial variables in PCO1 and PCA1 might yield further insight into the divergence of community composition between swab and biopsy samples in future work.

Despite being obtained from the same individuals, samples of shared-fish origin are not closely plotted in any multivariate analysis. Association of samples is observed to be largely due to sampling methodology, and not by individual of origin. This is in contrast to the results of Chapter 5, which suggested high individual variation between fish. The results of investigation of the adherent microbial communities seems therefore greatly impacted by sampling methodology, indicating greater impact on variation than from individual of collection or date of sampling. Permutational multivariate analysis of variance (PERMANOV) based on the Bray-Curtis dissimilarity matrices indicated significant differences in bacterial compositions between the two sampling methods ($p = 0.01$ in 999 unique permutations), with less significant difference between the two sample groups (8 and 9) represented in this dataset ($p = 0.021$).

6.4.2 Individual variation of microbial composition at different taxonomic levels with different sampling methodology

When total identified ASV's were contrasted between swabs and corresponding gill biopsy samples, a subset of taxa was identified by both methodologies, but an overall greater number were not (**Figure 6.4**). A total of 73 ASVs were concurrently identified in both biopsy and swab-derived samples, whereas 45 were observed exclusively within the biopsy samples, and a total of 230 ASVs were identified in swab-derived samples that were not seen in the biopsy samples. Approximately half of swab derived ASV's not seen in biopsies were taxa also identified in water samples, indicating that these microbes might be transiently present bacteria within the mucus layer of gills. The other half (126 total), however, were not identified in either biopsy samples or environmental water controls, only swab-derived samples. These represented microbial taxa present on the gills that biopsy-derived sequencing failed to identify. Similarly, biopsies identified microbes that swabbing failed to identify.

Figure 6.4 Shared and unique ASV's

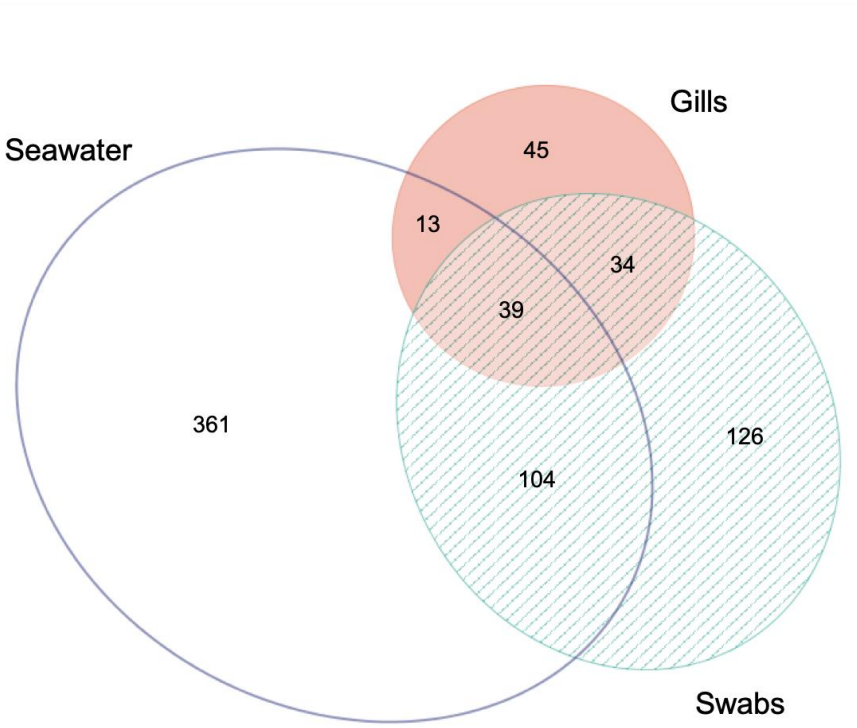


Figure 6.4: *Euler (Venn-type) diagram illustrating shared and uniquely identified ASV's from corresponding swab, biopsy and water samples.*

6.4.2.1 Dominant phyla vary between swab and biopsy derived samples

Four dominant phyla were identified within swab samples, in contrast to the six dominant phyla observed through identical analysis of gill biopsy samples (with dominance determined as average abundance of 0.5% or over within a community). The dominant phyla of swab-derived sampling were Proteobacteria (average abundance 72.8%), Bacteroidetes (22.3%), Chlamydiae (2.8%) and Verrucomicrobia (1.1%). The dominant phyla of corresponding biopsy samples were Proteobacteria (average abundance 87%), Bacteroidetes (5.7%), Chlamydiae (3.8%), Verrucomicrobia (0.7%), Firmicutes (0.7%) and Actinobacteria (0.5%). Statistically significant differences were identified between the relative abundance of Bacteroidetes in swab and biopsy-derived

samples (**Figure 6.5**). Based on this figure, abundance of Firmicutes within swab and biopsy-derived isolates was also potentially of interest, although not statistically significant. The relative abundance of Firmicutes in relevant gill biopsy samples was an average of 4.48% and 0.29% in total biopsy samples of Groups 8 and 9 respectively, whereas the average abundance of Firmicutes observed in swab-derived samples from Groups 8 and 9 were 0.02% and 0.12%. This variation in abundance suggests a difference in isolation of Firmicutes with different sampling methodology. Individual comparison of Firmicute abundance across sampling methodologies in the same fish demonstrated consistently lower Firmicute abundance from swab-derived results (**Table 6.2**). Firmicute abundance within a sample is proportionally greater in biopsy-derived samples 8F2, 8F4, 8F5, 8F6 and 9F2. The remaining samples either failed to identify Firmicute presence using both sampling methods, or a very low abundance (<0.1) was observed.

6.4.2.2 Variation in microbial proportional abundance obtained from individual fish with varied sampling methodology at Class level

Further to individual variation at the phylum level, variation was observed at lower taxonomic levels between swab and biopsy samples from the same source. Many publications relating to the adherent microbial communities of fish utilised stacked bar-plot figures for representation of relative community composition within groups of interest. Whilst these figures were not used to illustrate group averages in Chapter 5 due to the high individual variation in samples, they do provide interesting insight into samples on an individual basis when comparing sampling methodology. Figures were therefore created to observe differences in results obtained between swabbing and biopsy methodology for individual fish. Samples 8F2, 8F3, 8F4 and 9F2 are provided within the text for illustrative purposes (**Figure 6.6**). Plots for remaining fish 8F5, 8F6, 8F7, 8F8 and 9F6 are provided in appendix materials (**Appendix D.2**). Results of comparison of swab and biopsy-derived microbial populations demonstrate a clear difference in community composition obtained from different sampling methodologies at the Class level (**Figure 6.6**). General trends include a tendency towards dominance of Betaproteobacteria in biopsy-derived samples, alongside greater proportional abundance

of Chlamydiae, Bacilli and Deltaproteobacteria. Swab-derived results appear to contain a more even proportional abundance of observed taxa, with greater relative compositional contribution from Gammaproteobacteria, and Alphaproteobacteria. Individual variation can be observed in both swab and biopsy results. Although these figures are a common method for illustration of results in studies investigating microbial community composition, they do not provide information regarding the total number of ASV's obtained for each microbial class. This information is provided for the apparently proportionally variable microbial classes listed above, to enhance understanding of the results (**Table 6.3**). Information illustrated in **Table 6.3** demonstrates that despite the generally greater number of reads obtained from swab-derived samples, biopsy-derived samples consistently identified a greater number of sequences for Betaproteobacteria and Bacilli. This information greatly enhances the reliability of conclusions drawn regarding the proportional dominance as well as physical presence of taxa within these Classes.

Figure 6.5 Phylum and order level abundance

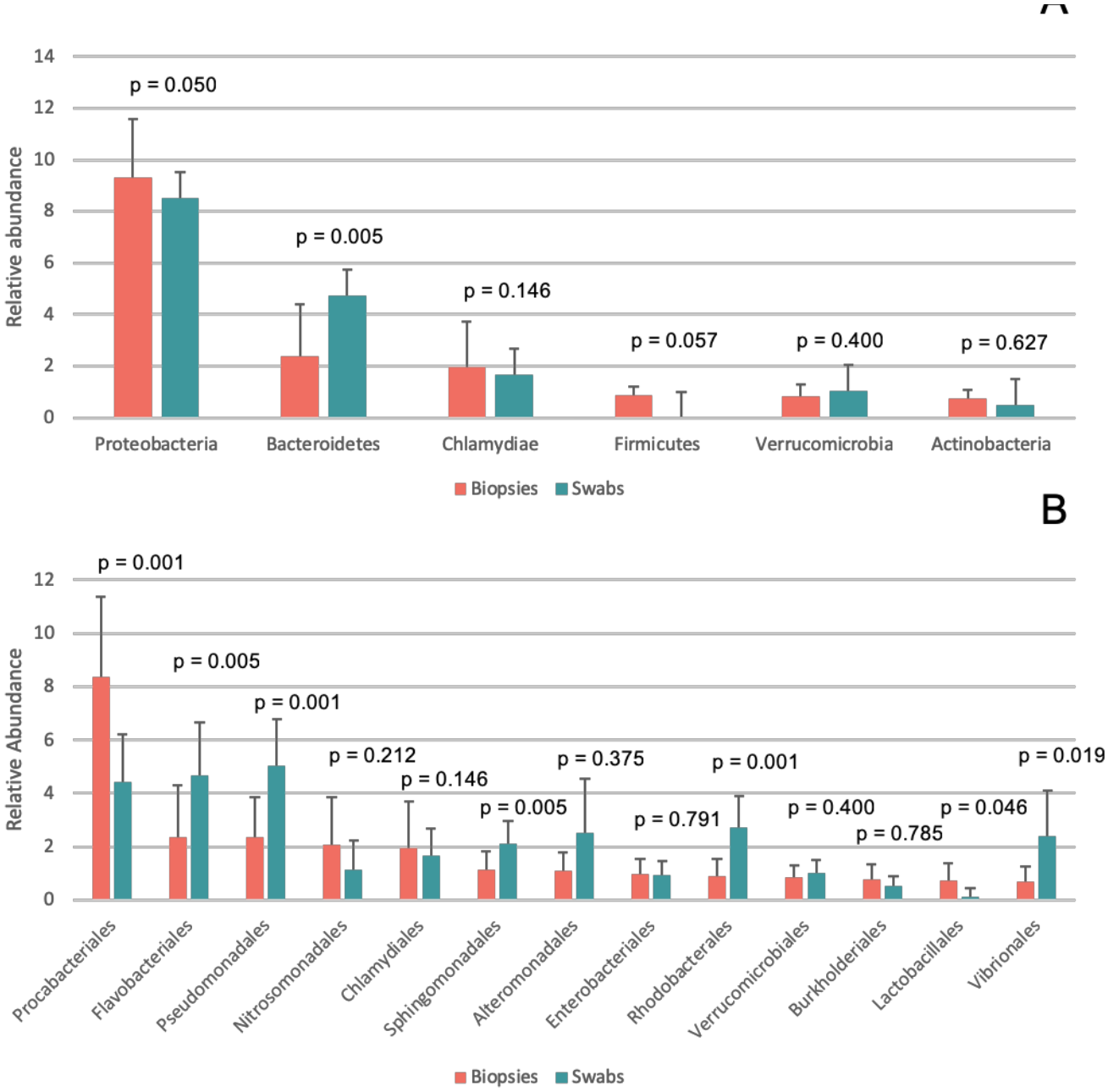


Figure 6.5: *Taxonomy results for classification above >0.5% abundance at phylum (A) and order (B) level from square root transformed data for biopsy (coral) and swab (viridian) samples. Error bars in bar plots represent standard error of the mean. Significance testing was performed using Mann-Whitney-Wilcoxon Test unpaired t-tests, with p values provided above bars.*

Table 6.1 Firmicute abundance

Group	Individual	Sample type	Relative abundance (%)
Group 8	Sample 8F2	Biopsy	3.91
		Swab	0.00
	Sample 8F3	Biopsy	0.00
		Swab	0.00
	Sample 8F4	Biopsy	0.25
		Swab	0.00
	Sample 8F5	Biopsy	0.58
		Swab	0.06
	Sample 8F6	Biopsy	0.23
		Swab	0.00
	Sample 8F7	Biopsy	0.00
		Swab	0.03
	Sample 8F9	Biopsy	0.58
		Swab	0.07
Group 9	Sample 9F2	Biopsy	1.11
		Swab	0.14
	Sample 9F6	Biopsy	0.00
		Swab	0.10

Table 6.1: *Relative percentage firmicute abundance in swab and biopsy derived samples for comparison of community composition across sampling method.*

Table 6.2 Bactereidete abundance

Group	Individual	Sample type	Relative abundance (%)
Group 8	Sample 8F2	Biopsy	2.8
		Swab	21.5
	Sample 8F3	Biopsy	3.3
		Swab	19.4
	Sample 8F4	Biopsy	35.0
		Swab	34.2
	Sample 8F5	Biopsy	1.5
		Swab	20.5
	Sample 8F6	Biopsy	3.3
		Swab	30.8
	Sample 8F7	Biopsy	0.9
		Swab	38.4
	Sample 8F9	Biopsy	3.0
		Swab	22.9
Group 9	Sample 9F2	Biopsy	0.9
		Swab	6.0
	Sample 9F6	Biopsy	0.8
		Swab	6.7

Table 6.2: *Relative percentage Bacteroidetes abundance in swab and biopsy derived samples for comparison of community composition across sampling method.*

Figure 6.6 Class level stacked bar for comparison by individual

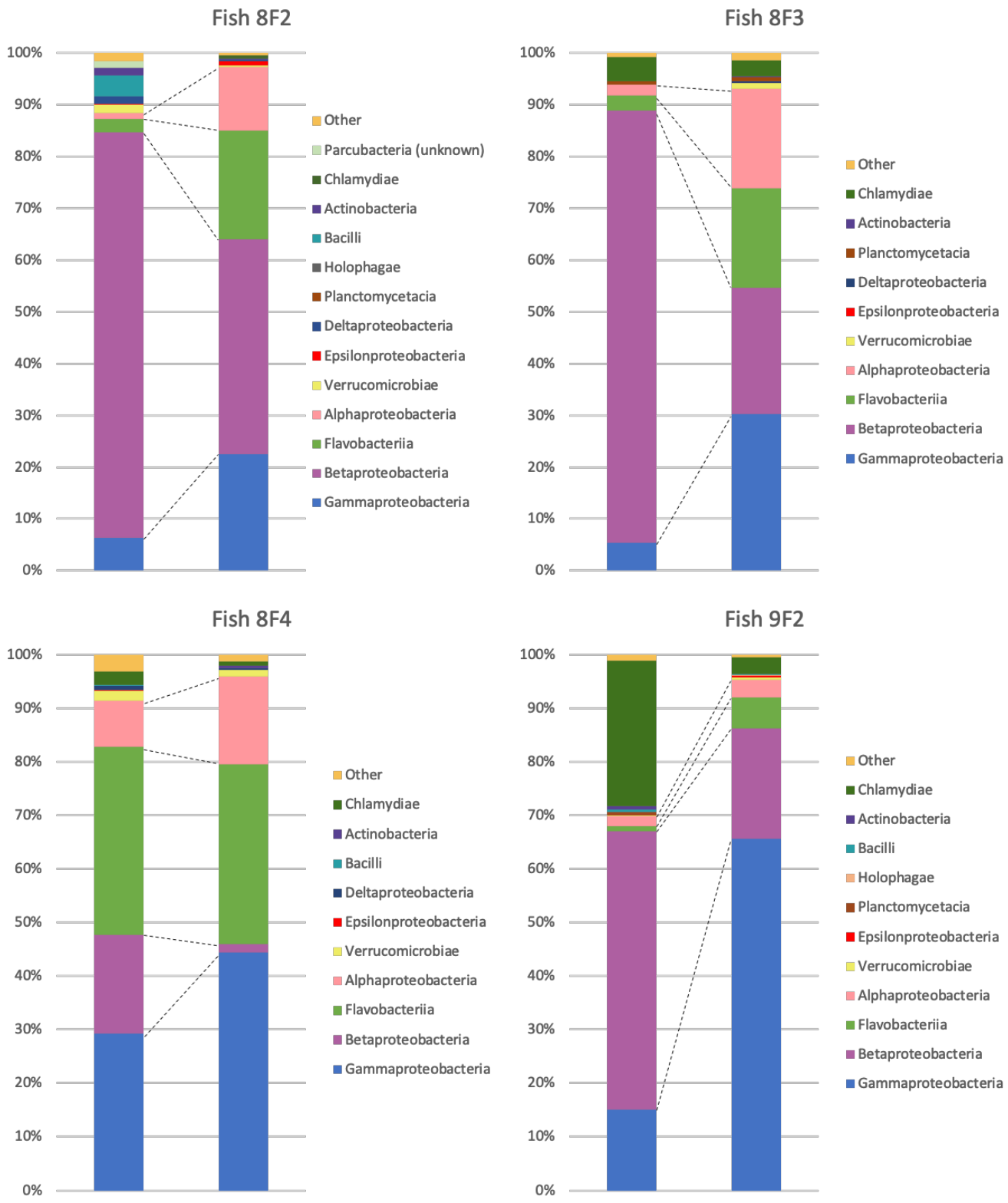


***Figure 6.6**: Stacked plot of community composition results of biopsy (left) and swab (right) samples from individual fish 8F2, 8F3, 8F4 and 9F2, classified to class level. Results for five additional fish are provided in Appendix D.2.*

6.4.2.3 Specific genera differentially isolated by swabbing and biopsy collection

Based on phylum and class level results, it is apparent that differences exist in proportional representation of microbial taxa with varied sampling methodology. Comparison of average abundance at Order level identifies a number of significantly differently abundant taxa between swab and biopsy-derived samples (**Figure 6.5**). Procabacteriales were determined by Mann-Whitney-Wilcoxon unpaired t-testing as significantly more abundant within biopsy samples than swabs. This order constituted an average of 69.8% abundance in biopsy samples relative to 19.5% in swabs. Significantly greater abundance of Flavobacteriales, Pseudomonadales, Spingomonadales and Rhodobacterales were detected in swab samples relative to biopsies.

Similarity percentage analysis (SIMPER) was also utilised in investigation of differences in the relative abundance of bacteria at genus level. A total of 17 ASV's accounted for 49.98% of observed variance between swab and biopsy samples. The microbiota identified with greatest contribution to dissimilarly between groups were (in order), *Procabacteriaceae*; unclassified (8.23%), *Psychrobacter* (6.37%), Flavobacteriaceae; unclassified, (4.52%), *Candidatus Piscichlamydia* (2.93%), Rhodobacteraceae; unclassified (2.79%), *Loktanella sp* (2.79%), Chryseobacterium (2.68%) and *Candidatus Branchiomonas* (2.56%). Of these, *Procabacteriaceae* and *Candidatus Branchiomonas* demonstrate greater relative abundance within biopsy samples, and the remaining genera are of greater abundance in swab-derived samples.

Table 6.3 Order level sequence counts

	Betaproteobacteria		Chlamydiae		Bacilli		Deltaproteobacteria		Gammaproteobacteria		Alphaproteobacteria		Total sequences	
	Biopsies	Swabs	Biopsies	Swabs	Biopsies	Swabs	Biopsies	Swabs	Biopsies	Swabs	Biopsies	Swabs	Biopsies	Swabs
8F2	**1403**	838	0	12	**70**	0	**28**	7	113	457	20	250	1790	2024
8F3	1536	1981	86	251	0	0	0	22	100	2442	40	1554	1841	8088
8F4	**290**	77	**39**	35	**4**	0	11	15	460	2230	135	824	1573	5026
9F2	**3248**	1473	**1696**	229	**29**	10	0	0	936	4695	113	235	6243	7161

Table 6.3: *Counts of obtained sequences from biopsy and swab samples from fish illustrated in stacked bar plots (Figure 6.6). Instances of greater overall sequence count for each class are highlighted in* ***bold*** *to emphasise the methodology where number of sequences in one methodology exceeds the number in the other.*

Figure 6.8 Before and after filtration

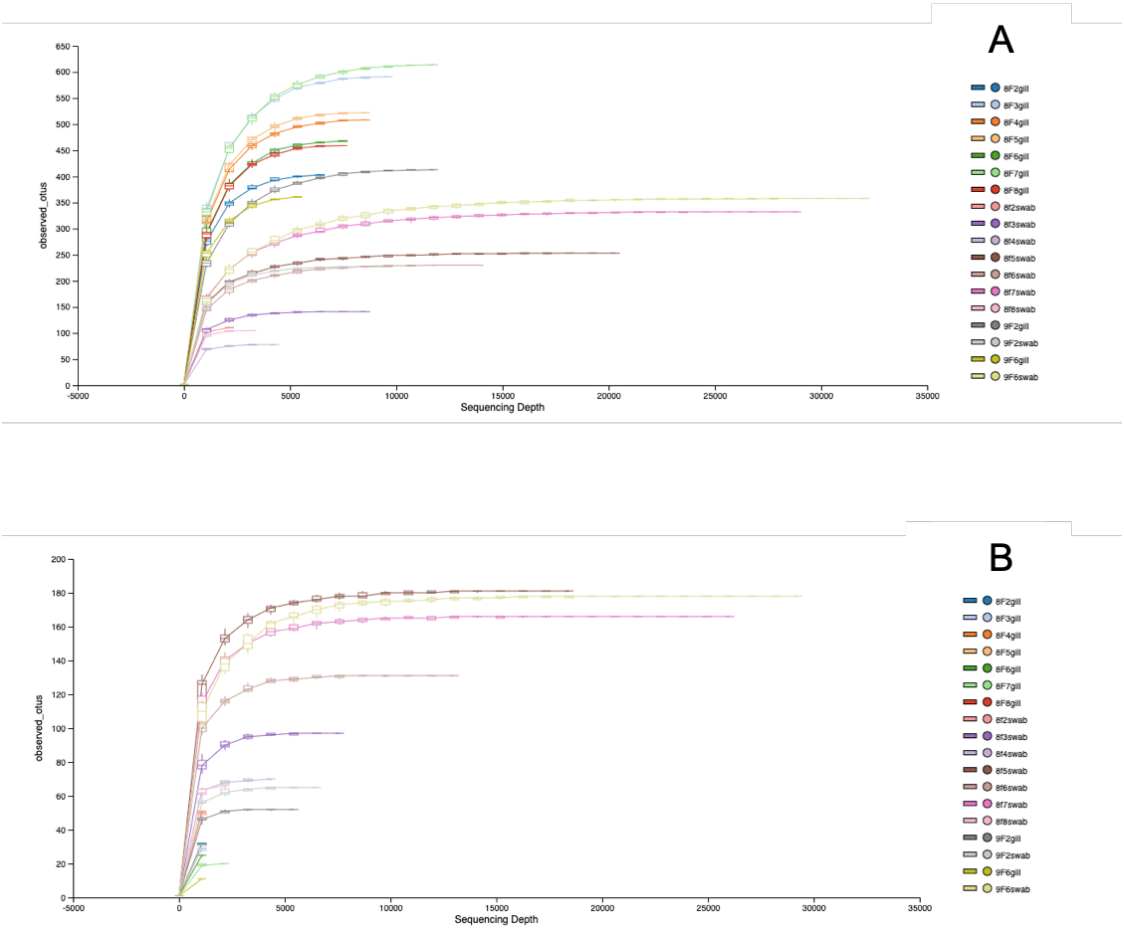


Figure 6.7*: Rarefaction curves before (A) and following (B) filtration to remove taxonomic assignations out-with desired 16S microbial SILVA results. Unassigned ASVs of non-bacterial origin are filtered in B.*

6.5 Discussion

This study was conducted with the aim of contrasting microbial communities from identical sources to determine if sampling method impacted analysis of community structure and, if so, provide recommendations for which method might best be employed in different contexts.

6.5.1 Study Design

6.5.1.1 Sample collection and DNA extraction

The study was designed to treat samples identically in every way except method of collection, so results might be contrasted to investigate this variable. Swabbing was conducted over an area of gill tissue approximately equivalent in size to the total sections of tissue removed and fixed during biopsy collection, only from the contralateral gill. Use of the DNeasy Blood and Tissue kit for DNA extraction, however, necessitated use of relatively small sub-sections of fixed gill tissue, equating to less than the overall front-facing gill area sampled by swabbing. Gills are multi-dimensional surfaces with a high overall surface area for gas exchange and other functions, and so it was not possible to quantify the 'true' gill surface area sampled using biopsies for comparison to swabs. Swabs were, however, potentially sampling a greater surface area, at least on the front-facing surface of gill tissue. It is therefore possible that use of swabs allows a greater gill surface area to be assessed when investigating the adherent microbiota. Previous research has demonstrated variation in obtained microbial diversity with varied tissue area sampled (Larsen et al., 2014). Intended as a pseudo-biological replication step, pooling biopsy derived DNA increased sampled surface area of gills.

A number of previous studies employed a wash step prior to swabbing the gill, with surfaces initially washed either with sterile water or ethanol to remove non-adherent populations (Al-Harbi and Uddin, 2008). In other work, researchers have deliberately removed all the mucus from fish prior to biopsy excision in order to study the skin

microbiota (Merrifield and Rodiles, 2015). In this study, gills were sectioned and swabbed directly, as it was considered that a wash step might displace mucus from the gill tissue since previous research showed that washing can remove biofilms (Palestrant et al., 2004). Removal of mucus is considered undesirable here, as it is a key component of gas exchange and microbial defence in fish (Koppang et al., 2015), and so might harbour essential components of the microbiome.

6.5.1.2 Amplification and sequencing

Biopsy extractions and swab-derived DNA were identically treated for amplification of 16S and preparation for sequencing as described in the Methods. Next-generation sequencing of swab and biopsy-derived microbial material provided an initially similar depth of sequencing prior to filtering. However, notably fewer ASV's are detected in biopsy-derived samples following processing for removal of unassigned and contaminant genomic material (**Figure 6.7**). Likely due to the high level of Atlantic salmon genomic material obtained through DNA extraction of biopsy samples, resulting in an overall lower input concentration of microbial DNA and undesired amplification of *Salmo salar* DNA by 16S primers. Investigation of the taxonomic assignation of the most frequently observed filtered sequences from biopsies using BlastN against the Genbank database determined that many of the unassigned and filtered reads from biopsy-derived samples were of salmonid origin. Although initial DNA extractions in swab and biopsy-derived samples were of similar concentration, the biopsy samples will have contained a great deal more host genomic material. This will have impacted the amount of microbial genomic DNA used in the initial PCR reactions, as well as masking of PCR reagents by Atlantic salmon DNA likely also inhibiting amplification of microbial 16S sequences.

An alternative to pooling of biopsy-derived samples prior to PCR might instead have involved individual PCR of separate extractions, and subsequent pooling to maximise available microbial DNA for use in Illumina sequencing. This was not considered at the time as it would have proved time-consuming due to the requirement for three times as many initial PCR reactions, however this approach would likely have enhanced the

results. Alternatively, inclusion of an initial PCR step for amplification of the 16S gene with universal primers in a nested PCR protocol might have reduced the level of host DNA contamination, as would inclusion of blockers. This was considered and rejected in the original experimental design due to the associated bias in final library composition with additional PCR reactions. However, in hindsight, results of the sequencing of isolates in Chapter 5 might have benefitted from inclusion of this step to improve microbial sequencing depth.

Salmonid DNA inclusion was obviously a problem associated with use of gill biopsy samples. Assessment of the rarefaction curves implies that despite the low sequencing depth of processed biopsy-derived reads, sufficient depth was achieved to capture the diversity of the PCR product. However, this relates only to the microbial material that was successfully amplified and fails to account for poor initial PCR yields. Based on high host genomic material inclusion, it is suspected that a lack of microbial DNA was included in samples from biopsy extractions. Low microbial genomic input to PCRs and failure to amplify the complete microbial diversity by primers due to masking from high host DNA amplification must therefore be considered. Similar studies investigating the microbial community obtained by swab or biopsy excisions in humans also highlight generally lower microbial richness within biopsies (Araújo-pérez et al., 2012). Previous publications in human medicine suggest that sub-surface tissue layers are of lower microbial diversity and richness than surface epithelium, however this result must be viewed with a degree of scepticism, as the technique of biopsy excision here might also have impacted results through failure to capture the full diversity present.

6.5.2 Community composition with varied sampling methodology

Based on the findings of this research, sampling methodology appears to have a significant impact on the assessment of community composition at multiple taxonomic levels. The greater number of swab derived ASV's obtained following filtering suggests a greater overall efficiency in microbial surveying by swabbing. However, the 45 unique ASV's obtained from biopsy-derived samples demonstrate the utility of biopsies in obtaining a number of microbes that swabbing fails to identify. It is possible that techniques such as swabbing obtain populations from the surface of tissue, allowing

sampling of larger areas than biopsies, but may fail to obtain microbiota identified by biopsies. Conversely, biopsies are destructive and allow assessment of only a limited sample area, with greater proportional host DNA contamination. Biopsies appeared to excel however in the isolation of cryptic or even intracellular microbes by sampling intradermal areas and surface gill tissue that cannot be reached by swabbing alone. Previous research in the study of human skin (Grice et al., 2008) and rectal tissue (Araújo-pérez et al., 2012) reached similar conclusions, that swabs and biopsies achieve different microbial coverage.

An important consideration however is in the potential for intra-individual variation in microbial populations. Swabs and biopsies were obtained from the same individuals; however, they were taken from contralateral gills. Samples were obtained from the first gill on the left (biopsies) and right (swabs) side of the fish. This is important to keep in mind when assessing community composition as previous studies have noted divergence in community composition even within tissue compartments, where even closely associated structures can differ in their adherent microbiota. Although the first gills perform the same function with similar demands on their microbial symbionts, and likely experience similar stresses, contralateral gills are not necessarily identical. A number of structural deformities were noted within the study population (see Chapter 5), including abnormal gill structure and shortening of the opercula. Structural deformities, incidence of trauma or unilateral gill disease are all potential factors that might cause heterogenous microbial communities between left and right gills of fish. Fish with visible deformities were not sampled in this chapter for this very reason, and neither were fish with divergent gross pathology in either gill, or apparent clinical signs above that routinely observed within the population. However, although this study sought to address this issue insofar as was possible, the potential for microbial communities to truly differ across contralateral gills cannot be excluded.

6.5.2.1 Use of biopsies

Biopsies were utilised to obtain bacterial genomic material for the bulk of next generation sequencing conducted in this thesis. The results of Chapter 5, investigating

the association of gill pathology with adherent microbial communities, are entirely based on biopsy-derived samples. The decision to utilise biopsies was made based on a review of the literature which suggested the presence of 'cryptic' microbial populations (including between lamellae or associated with inter-branchial cartilage as well as within subepithelial tissue layers) and on the pathogenesis of various gill diseases. Biopsies were obtained as it is suggested in human research that biopsies allow sampling of a broader array of structures than can be accessed by swabbing.

Results of both alpha and beta diversity demonstrate clearly divergent community composition obtained by swabbing and biopsy excision. At the phylum level, biopsy sampling appears consistently more successful in isolation of taxa within the Firmicute phylum. Firmicute bacteria are generally gram-positive staining, and many can produce endospores, conferring resistance to desiccation and survival of extreme conditions (Hutchison et al., 2014). Firmicutes are consistently identified in the core microbiota of fish species (Ahj Van Kessel et al., 2011; Lokesh and Kiron, 2015; Ornelas-García et al., 2018), and their identification is therefore crucial not only in targeted studies, but also as part of the adherent microbiota, as part of understanding microbial community composition and their symbiotic role in fish health. For example, the Firmicute genus *Epulopiscium* is the subject of much research focused on the gut microbial community of sturgeon fish, and is hypothesized to be a symbiont that can form endospores as a mechanism for nutrient conservation during periods of fish fasting (Clements et al., 1989; Clements and Bullivant, 1991; Flint et al., 2005). Microbiota identified exclusively through biopsy sampling included Clostridial microbes, a class of Firmicutes that include bacterial species known to be harmful to fish (Gao et al., 2013). Since these bacteria are obligate anaerobes, conditions in areas of the gill that cannot be sampled by swabbing, such as beneath the epidermal layers, might be more favourable for this group. Oxygen concentration relative to the environment might be reduced in deeper tissue layers, or within tissue folds and layers not readily exposed to the environment. These results suggest that biopsy sampling has obtained microbial populations that cannot be accessed by swabbing, potentially within the tissue itself.

Much is still to be learnt about the microbiota of fish, particularly in the gill. Failure of swabbing to identify a number of Firmicute genera that were found in biopsy samples highlights the information that can be missed when swabbing methodology is used alone. Biopsies sampled an entire cross-section of gills and it appears as though there are components of the microbiome present that were not obtained by swabbing.

Based on various analyses including SIMPER, multivariate analysis and significance testing of divergent abundances between sampling methodology (**Figure 6.5**), a number of microbial taxa were identified as contributing to the observed variation between communities. Biopsy samples contain significantly greater *Candidatus Branchiomonas* and *Procabacteriaceae* populations, with sampling using this methodology not only resulting in proportionally greater relative abundance of these microbes, but also consistently higher total sequence counts. Little is known about the single genus containing family Procabacteriaceae, other than the fact it is a documented obligate intracellular microbe of *Acanthamoeba*. *Acanthamoeba* are distantly related to the organism *Paramoeba perurans,* which current research indicates is responsible for the serious gill disease in farmed Atlantic salmon, AGD (Stene and Aspehaug, 2017). There is limited research regarding the association of *Acanthamoeba* with disease in fishes, however asymptomatic fish are reported in the freshwater environment (Dykova et al., 1999), and acanthamoeba is a documented pathogen of goldfish (Voelker et al., 1977). *Candidatus Branchiomonas* is an intracellular bacterium associated with the microbial disease epitheliocystis in fish (Herrero et al., 2018; Toenshoff et al., 2012). Epitheliocystis is an emerging disease in Atlantic salmon production, although some research suggests it has been an underlying undiagnosed condition for some time (Downes et al., 2018; Isabel et al., 2018). Cystic lesions within the gills of impacted fish contain chlamydia-like organisms and are thought to reduce the functional ability of afflicted fish through damage to gill tissue, making them a serious concern in aquaculture production of salmonids and many other fish species (Bradley et al., 1988; Nylund et al., 2015; Paperna, 1977). Based on the results of this research, the assessment of community composition from biopsies is greatly impacted by the presence of *Candidatus Branchiomonas*. From these results alone, it is not possible to determine whether this microbe is a normal sub-epithelial component of the microbiome

of gills, or if its isolation here is an indicator of dysbiotic change, although the results of Chapter 5 do suggest an association with gill pathology. The greater prevalence of this bacterium in biopsy samples does though support the assertion that this is an intracellular bacterium, and perhaps suggests an advantage of biopsy sampling if this bacterium is of interest. Previous studies have also involved taking gill sections for its isolation, however this previous work provides no details about the tissue sections taken or relative success in sampling with other methods (Toenshoff et al., 2012).

6.5.2.2 Use of swabs

Swabbed samples in this study were collected purely for the purposes of comparison to biopsy samples. Swabs are routinely utilised in the study of the adherent microbiota of skin and gills in fish, with the majority of papers using swabs rather than biopsies. Biopsies were considered a more appropriate method of collection of microbial communities for the work conducted in Chapter 5 specifically however as it was focused on various disease states of gill tissue, as a number of gill pathogens are known to be intracellular. It was therefore of interest to compare community composition and the obtained microbes from biopsies and swabs to determine whether results from these methodologies can be compared across studies, and if not, which method is optimal in the study of the gill microbiome.

Comparison of community composition between swab and biopsy samples of individual fish demonstrated marked differences in proportional abundance at multiple taxonomic levels. Total obtained reads in swabs exceed those obtained in biopsies across individuals for all classes with the exception of Betaproteobacteria (**Table 6.3**). Stacked bar charts for individual fish demonstrate trends towards a dominance of certain classes in biopsy-derived samples, with greater proportional abundance of Betaproteobacteria, Chlamydiae, Bacilli and Deltaproteobacteria, while swab-derived results appear to contain a more even proportional abundance of observed taxa, with greater relative compositional contribution from Gammaproteobacteria, and Alphaproteobacteria. It appears therefore that the lower richness captured from biopsy samples might bias proportional abundance for apparent dominance of a small number of classes. These

apparent trends in community compositions are supported by the results of alpha diversity measures (**Figure 6.3**). Swab-derived samples appear more diverse and with greater species richness than concurrently obtained biopsies. This may be due to a number of factors, including greater environmental contamination as well as the technical issues associated with biopsies. Appraisal of shared and unique ASVs (**Figure 6.4**) demonstrates a greater number of identified taxa from swab results shared with environmental populations. Given that the area sampled by swabs was the gill surface, and the majority of biopsy tissue was not exposed to environmental communities, the potential bolstering influence of environmental microbes through greater 'contamination' of swabbed samples with environmental microbes might be considered. The nMDS results and assessment of shared ASV's across sampling methodology indicates that swab-derived samples more closely resemble those of environmental water populations, however this does make sense biologically. Naturally shared microbiota between adherent microbial communities and the environment is of course likely though, due to the recruitment of bacteria to gills from these environmental populations, and a lower diversity of subepithelial microbiota might reasonably be expected. Historical research incorrectly suggested that only cryptically located structures of the gill might host specific microbial communities, however this research supports more recent publications that identify the microbiome of gill tissue obtained by swabbing as distinct from environmental populations.

Based on an extensive literature review, it was determined that the majority of publications in this field have used swabbing as their method for sample collection for assessment of the microbial community composition of fish gills. The results of this research suggest that the microbiome of the gill surface is richer and more diverse than that obtained by biopsy excision (which samples deeper tissue layers and more cryptic locations, in addition to a small region of the surface community). This may be due in part to the greater presence of environmental microbes on the gill surface, for the consequent preferential inclusion of these environmental microbes within swab-derived samples. The current results do, nevertheless, suggest that whilst swabbing may lead to underestimation of intracellular microbes and fail to capture those localised beneath the surface mucosal layer, swabbing does capture a greater richness and diversity of

microbiota overall. Use of biopsies allows sequencing of these more cryptic microbiota, however, biopsies also fail to capture the full microbial diversity that is present.

Informative future avenues for this research might include scanning electron microscopy to determine whether microbial abundance truly differs in different regions of the gill tissue surface, or use of FISH to observe microbial taxa *in situ*.

6.5.3 Indications for different sampling methodologies; the argument for use of swabs and biopsies to answer different biological questions

Results of this research demonstrate a clear difference in the assessment of microbial community compositions of swab and biopsy samples. Swabbing obtains a richer, more diverse microbial community coverage without suffering from the processing complications of host DNA contamination observed with biopsy samples. However, swabbing remains useful only in the assessment of the surface microbial communities. Results of sequencing swab-derived samples identifies a number of taxa also identified in analysis of microbial populations of saltwater samples (environmentally derived populations). Whilst some bacteria that are commensals on the gill are also likely present in the environment, there is also the possibility that the swab samples could be contaminated with bacteria with no biological relationship with the gills. Some previous research has extoled the benefits of rinsing tissue prior to sampling, specifically to avoid this kind of contamination with non-adherent populations. However, it was the considered opinion of the author that rinsing might dislodge mucus and microbes from the gill surface and compromise results, so this step was not included. Future studies might wish to include this step when performing gill swabs, although mucus is likely an important location for the microbiota. Relative to results of biopsy-derived samples, swabs demonstrate a greater proportional contribution to community composition from Alphaproteobacteria and Gammaproteobacteria. Alphaproteobacteria are largely phototrophic microbes, but Gammaproteobacteria are a class of microbes containing a number of salmon pathogens, including a number of the potential pathogens identified in previous chapters (*Vibrio*, *Pseudomonas* and *Aeromonas*). The action of these microbes in erosive pathologies likely makes them accessible by swabbing, as used in

previous research for their identification from fish gills (Cahill, 1990). Interestingly, *Serratia* (considered an opportunistic pathogen in some conditions) was identified in all swab-derived samples, but only 7 out of 9 biopsies. *Pseudomonas* was identified in 8/9 swab-derived samples, but again, in fewer of the biopsy-derived samples, and *Tenacibaculum* was identified only by swabbing. Swabbing is selectively sampling from the mucoid layer, and so results suggest that this layer might by the location for these taxa.

It is also well documented that various pathogens of salmonids can be intracellularly located. Thus, depending on the research question of interest, swabs may not be sufficient for studying the gill microbiome. Based on these results, a difference in the utility in sample collection by use of swabs or tissue biopsies is apparent. Swabs appear useful in identification of greater overall microbial diversity, but with the potential risk of inclusion of a greater proportion of environmental taxa. They appear more successful in identification of gammaproteobacterial commensal pathogens, as well as alphaproteobacterial taxa. Swabs are non-destructive, allowing sampling without the need to euthanise fish, although repeated swabbing is not recommended as it would likely introduce variables involving mucus removal and microscopic tissue trauma to gills. Gill biopsies appear more successful in identification of intracellular microbiota, and potentially sample cryptic locations that cannot be accessed by swabbing, however are problematic to process and fail to capture much microbial diversity.

6.6 Conclusion

This research demonstrates a measurable difference in the microbiome coverage between swabs and biopsies obtained from the gill tissue of the same Atlantic salmon individuals. Despite a limited sample size, significant differences were identified within the dataset when comparing sampling methodologies. Although environmental factors and disease could not be controlled in fish obtained from the marine environment, no statistically significant impact was detected for additionally measured variables. Samples show a clear association based on sampling methodology over individual variation, indicating that the method through which microbiota are obtained has a large influence on final results. These results potentially reflect a variation in resident microbiota of different anatomical sites, with differences detected between superficial swabbing or full thickness gill biopsies of tissue, notwithstanding the limitations of the methodologies in detecting the microbes present.

There is a lack of consensus in the literature regarding the comparability of swab and biopsy samples from different human tissues, with an apparent importance of site of interest. No such study has been conducted to our knowledge on the impact of sampling methodology on 16S sequences obtained from fish. These results therefore represent an important first step in optimising the study of the microbiomes of commercially important finfish like Atlantic salmon, with clear potential for extrapolation to other salmonid species and possibly beyond. Results varied in a number of ways, including altered dominance of phyla and presence of variable genera. The results of biopsies indicate the sub-epithelial microbial communities might be overlooked or at least proportionally under-represented when only surface swabs are obtained. Intracellular pathogens in particular are of concern in the aquaculture production of salmon, with these results indicating that biopsy excision should be used rather than swabbing for the isolation of these microbiota. Nevertheless, swabbing samples only from the surface microbiome likely captures a greater number of mucoid and environmental microbiota, as it can be performed over a larger area than that extracted from biopsy tissue. Results indicate a closer association with environmental populations for surface and mucoid

microbial communities, with greater beta diversity observed in samples obtained by swabbing.

It appears therefore that careful consideration must be made when designing studies of the microbiome in fish gills, and selection of a sampling technique must be tailored to the question being asked. Swabs and biopsies appear to have both positive and negative aspects for consideration, when choosing the method of collection for bacteria of the adherent microbiota, and despite the prevalence of swab use in published literature, indications so exist for biopsy collection in subepithelial analysis and pathogen diagnosis. The decision to collect swabs, biopsies or even both must depend not only on technical ease but also on the hypothesis to be tested. With increasing use and applications for metagenomic techniques such as 16S next-generation sequencing, standardization of collection and processing methods will aid in interpretation and comparability of results across studies. Study design must vary, however, depending on the scientific goal of the research, particularly if the goal is for characterisation of the adherent microbiota of gill tissue beyond just the surface and mucoid communities. Previous research has identified sample fixation, storage and method of DNA extraction as having a significant impact on the study of the microbiota of fishes. The current research reveals that the method of sampling is also a major concern.

Chapter 7

7. Discussion

Globally, salmonids account for the greatest profit of any fish harvest sector, both in their aquaculture production and through wild fisheries (FAO, 2016b). Gill disease has emerged in recent years as an important limiting factor in the production of fish, with impaired health status having various knock on effects, including reduced welfare, reduced production efficiency, reduced survivability and an overall reduced output and profit from the industry. It is therefore in the best interests of the industry to prioritise improving the gill health status of stock. Gill conditions are particularly problematic in the marine-cage style of salmon aquaculture production due to exposure of the fish to mixed infectious and other harmful environmental organisms. Although much research has focused on answering important questions of how to prevent and control infections, there are a number of environmental factors that have been shown to have the potential to negatively impact gill health that are relatively understudied. Gills are delicate organs and disruption of homeostasis can have negative consequences for fish, irrespective of infective or non-infective cause. Little information is available regarding the impact of cnidarian jellyfish and harmful algae on gill trauma and impaired fish health. Research into the role of these non-infectious factors but still harmful factors in facilitating initial pathology and subsequent predisposition to infectious disease is currently lacking. This thesis therefore sought to address the identified knowledge gap.

Research suggests that exposure to cnidarian jellyfish and phytoplankton in salmonids might result in predisposition to various pathologies, and while the exact mechanisms are unclear, immunological and structural changes to gills are known to result in impaired functionality and predisposition to disease. An understanding of the impact of these organisms on fish health is crucial to the advancement of the aquaculture industry. Although Cnidarian jellyfish and phytoplankton will certainly present a control and mitigation strategy challenge, increased understanding of their impact might assist in monitoring and in novel control therapies. As well as their impact on gill structure and function, this thesis aimed to investigate the association of non-infectious harmful environmental organisms with impaired immunity and secondary infections. Studies of

complex gill pathologies and consequences of jellyfish exposure highlight a role of microbes in many infectious pathologies, and so microbial change was also considered of interest here as an important component in the assessment of fish health. Fish in the marine environment are exposed to many individual factors that might result in disruption of microbial communities, so the microbial communities associated with mixed pathology was explored. Disease can affect the adherent microbiota in many other organisms, and so gill pathology was investigated as a variable in microbial community restructuring. Overall, research was conducted to enhance the collective understanding of the direct impact of harmful organisms, the gill response of fish at the cellular level, and the downstream consequences of gill pathology in farmed fish.

7.1 Understanding the impact of harmful environmental organisms

7.1.1 Altered gene expression in response to phytoplankton exposure, not just a toxic chemical, not an infection.

Unlike an infectious agent, phytoplankton are associated with indirect harm to fish. Inducing harm either through their silicate cell structure, oxygen depletion or toxin formation, these organisms do not impact fish health through a classic disease process (Rodger et al., 2010). Relatively little is known about the response they illicit in gill tissue the fish they injure, particularly in response to algal toxins. Relevant existing studies in marine organisms are focused mainly on the impact of toxins from algae such as the dinoflagellates *Prorocentrum and Dinophysis*, and the risk they pose to human health through bioaccumulation in bivalves (Berdalet et al., 2015). *P. parvum* represents a phytoplankton of particular interest to finfish aquaculture, with an apparent action in gills (Watson, 2001), for which this kind of research was previously unavailable Large fish mortality events of both wild and farmed animals have been reported globally in association with blooms of this toxin-producing phytoplankton, making exploration of the transcriptome response an important area of study.

This study represents the first transcriptomic investigation of the salmonid response to *P. parvum* exposure and provides valuable insight into the action of its toxin through

analysis of the compensatory mechanisms employed by fish. The full toxin profile of this phytoplankton is yet to be characterised, and much of the literature can only speculate regarding the presumed action of toxic components (Henrikson et al., 2010; Kozakai et al., 1981). Many other toxins, such as effluents and heavy metals, or venom, have been demonstrated to have an immunosuppressive action in fish (Dunier, 1996; Thell et al., 2014), however this research demonstrated that *P. parvum* stimulates an immune response in fish gills. In terms of the compensatory activity of gill tissue, a marked antioxidant response was observed to occur in gills of rainbow trout exposed to high concentration of toxin producing *P. parvum*. Tissue response pathways to ROS, as well as activity in clearance of toxic protein components were detected. Antioxidant pathways of response previously observed in bivalves following exposure to other toxin-producing phytoplankton are considered a detoxifying response to both the protein component and reactive oxygen species of these harmful algae. Differently regulated transcripts associated with a response to oxygen deprivation and cell death support the hypothesis that, in addition to an internal toxic effect, *P. parvum* induce direct damage to gill surfaces too, with cell death and impaired oxygen uptake from traumatised tissue. Overall, results of this work suggest a similar mechanism of response in rainbow trout gills to *P. parvum* as occurs in bivalves to other toxic phytoplankton. A clear dose effect is seen with increased treatment concentration, but a generally shared functionality of response was recovered with *P. parvum* treatments. Further research might identify genetic predisposition to *P. parvum* tolerance or susceptibility in different rainbow trout groups, however based on available information in this study, the divergence of clinical presentations observed seems best explained by an unanticipated unequal level of *P. parvum* exposure in high concentration treated fish, and possible exhaustion of the acute stress response.

Phytoplankton are essentially ubiquitous in aquatic environments, with rapidly fluctuating populations that can be hard to predict and mitigate. The toxic effect of *P. parvum* is clearly dose dependant, and the clinical symptoms of low-level exposure might potentially go undetected in the farmed situation. These results demonstrate however that even low concentration exposure to *P. parvum* alters the immune response within gills, damages tissue, impacts gas exchange, and likely impairs overall gill

function. Phytoplankton like *P. parvum* could then feasibly be having an undetected negative impact on fish production, predisposing and acting in initiation or continued negative effect in multifactorial gill pathologies. A balance between host control and pathogen presence exists in gills, tissue that can appear perfectly healthy but still test 'PCR positive' for the presence of many infectious organisms. Factors that impair the defences, alter the homeostasis of gills or otherwise disrupt this balance provide an advantage to infectious organisms, and are associated with initiation or predisposition to disease.

Based on the results of Chapter 4, future research might now be able to better focus on the mitigation of toxin exposure in the farm situations, as well as inform producers as to predisposing factors to gill disease outbreaks. Further study must include the transcriptome response to other harmful phytoplankton, such as marine organism *Karenia brevi*s, on other aquacultured fish species. These results demonstrate that neither infectious agents nor inorganic environmental contaminants are a good model for exposure to the phytoplankton. This research might therefore prove seminal for further work interested in the impact of biological toxins such as those produced by phytoplankton, as one of the first of its kind conducted in exploration of the gill response in salmonids. Recent research in algal mitigation has advocated the use of hydrogen peroxide for the treatment of *P. parvum* (Wagstaff et al., 2018). Extensive anecdotal reporting from Atlantic salmon producers indicates that chemical treatment with H_2O_2 for sea lice or AGD is stressful for fish, and can result in high mortalities when fish are already experiencing functional impairment of gill tissue or undetected pathologies. Use of H_2O_2 for *P. parvum* blooms might therefore not only result in the destruction of phytoplankton, but also in the death of fish already compromised by the toxins of this algae. Perhaps a greater understanding of the action of this phytoplankton and cellular response of fish could avoid use of chemical treatments. Inactivating toxic components in the environment seems a possible avenue of mitigation, or the induction of some form of immunity in fish to reduce exposure impact. Studies are already underway in investigation of the potential for vaccination of fish against marine toxins (Camacho et al., 2007), an avenue that might be explored for protection too against *P.*

parvum and other toxin producing algae. This initial exploration of the tissue response to *P. parvum* might then aid future research into vaccine development.

Many aquaculture producers are currently monitoring algal populations in the marine environment, yet this information is only utilised in identification of high density HABs, associated with mass mortalities. Dissemination of the results of this research might inform the activities of producers, particularly regarding the risks of stressing of fish through handling or treatment of other gill pathologies in the presence of even low levels of harmful algae. All aquaculture activities must be considered with regards cost benefit balance, and the negative consequences to fish health from phytoplankton exposure exacerbated by surface feeding or sea lice treatments might far outweigh the potential benefits of increased productivity from activities such as H_2O_2 treatments for sea lice.

7.1.2 Impact of cnidarian jellyfish on fish health

Chapter 3 of this thesis involved assessment of the potential infectious consequences of jellyfish exposure to Atlantic salmon. This research demonstrates that, while the origin of secondary microbial infection of gills can occur from the environment or existing gill populations, jellyfish are capable of hosting a number of potentially harmful bacteria that might also present an infectious challenge to fish with jellyfish exposure. These results build on previous research, identifying additional infectious microbiota from the microbiome of cnidarian hosts, and suggesting the potential for previously unexplored jellyfish species to act as natural hosts of microbes and vectors of disease. Prior to this study, little research existed regarding potentially harmful microbes that might be present on the surface of jellyfish tissue beyond *T. maritimum*. Results of this thesis though identify a number of microbes including causative agents of furunculosis and vibriosis; *A. salmonicida* and various *Vibrio* that infect not only Atlantic salmon, but their susceptible cleaner fish cohorts too.

Although cnidarians can obviously sting both wild and aquaculture produced salmonids, they are thought to have a disproportionately negative impact on farmed fish

particularly due into their confinement, and subsequent inability to escape blooms. Cleaner fish like ballan wrasse and lumpfish are an important step in reducing use of pharmaceuticals such as H_2O_2 in Atlantic salmon production, by acting as a biological means to control sea lice. They can however be expensive to rear and environmentally costly to capture from the wild. It is essential therefore that their health following introduction to net pens be safeguarded. Research has shown that cleaner fish suffer from many of the same gill conditions as Atlantic salmon, including AGD, but it is bacterial disease that is most frequently implicated as the cause of mortalities. Unlike Atlantic salmon too, the vaccination protocol for these fish is still under development and they are considered particularly susceptible to bacterial infections (Nilsen et al., 2014), such as are caused by the microbes identified from jellyfish in this thesis.

Concerns regarding the impact of cnidarians are also usually focused on the ability of large blooms to effect mass fish kill events, however even microscopic cnidarians not previously associated with tissue trauma such as *N. pileata* have now been implicated in increased mortalities of farmed fish (Kintner and Brierley, 2018). This work identified potential pathogens from the tissue of three cnidarians present in coastal environments of geographical areas utilised for Atlantic salmon production. Microscopic *N. pileata*, sessile life stages of *O. geniculata* and *C. capillata* (known to directly traumatise gill tissue) were all found to host potentially pathogenic microbes.

Although the damage from at least large species of jellyfish has been well documented and is accepted now by the aquaculture industry as of concern to fish health, the monitoring and prediction of jellyfish blooms remains an arcane subject. Nets have been shown to be a functional growth environment for the sessile life stage of a number of cnidarian species, such as *O. geniculata*. These sessile life stages can also negatively impact fish health through stinging, and power washing of net pens has been shown to dislodge cnidarian polyps, allowing contact with fish (Bloecher et al., 2018; Fitridge et al., 2012; Guenther et al., 2010; Hodson et al., 1997). Despite this knowledge, aquaculture producers do not conduct water samples to monitor the level of cnidarian exposure within net pens. There exists no baseline data regarding 'normal', low or high population densities of any of these organisms in the marine environment. Anecdotal

reporting of larger species observed from the cage side represents the extent of population monitoring at the majority of facilities, with water sampling only conducted by visiting health professionals following clear mortality or gill pathology events. Reports of cnidarian impact on fish is therefore generally only in association with large bloom events, where investigation is conducted into the cause of mass die offs of caged fish, or from controlled-environment laboratory studies of single cnidarian species (Baxter et al., 2011b; M. D. Powell et al., 2018). Due to the nature of the biology of both phytoplankton and cnidarian jellyfish, retroactive sampling at farm sites is not helpful in linking density of exposure and the influence of these organisms on observed gill disease, as it fails to provide an accurate picture of populations when the damage was actually done. Phytoplankton populations can rise and drop throughout the period of a day with altered environmental conditions, and little is known about the factors that induce blooms of jellyfish. There therefore exists little published data regarding the association of cnidarian jellyfish with less dramatic gill pathology, a focus of study that would represent a crucial step in exploration of the factors that might predispose complex gill pathologies in farmed fish.

Phytoplankton species can be monitored using satellite imaging of chlorophyll in the environment, however jellyfish cannot be visualised this way, and although alternative methods such as acoustic recognition have been studied, they are not applied in the context of aquaculture protection (Brierley et al., 2005; Harvey et al., 2015; Hirose et al., 2009). Increased knowledge as to the potential pathogens present within the microbial community of jellyfish is of little use to aquaculture producers without an understanding of the presence of these jellyfish within their net pens. So overall, whilst an understanding of cnidarian association with potentially harmful microbes is of interest from a fish health standpoint, more research is required before this work can truly be applied. Only with accurate datasets that can be related to observed gill disease will the true influence of these organisms on fish health be understood. Jellyfish and phytoplankton may well be associated with the complex gill pathologies observed in many farmed fish, however their involvement cannot be confirmed without an understanding of their presence in the caged environment. Retroactive presumptive diagnosis by clinicians without accurate estimates of what are considered normal or

abnormal levels in the environment is not helpful. Sampling for zooplankton is relatively simple, similar to the phytoplankton sampling already being conducted on marine cages. It is proposed that to truly understand the impact of these organisms, careful and sustained water sampling must be conducted by aquaculture producers.

Direct transfer of microbes from jellyfish has yet to be demonstrated, and based on the results of this research, no comment can be made on the actual infective potential of the isolated microbes. Further research might then explore the potential for cnidarian jellyfish to transmit microbial pathogens in a controlled exposure trial with fish of known health status and cnidarians hosting pathogenic microbes within their surface communities. Demonstrating the transfer of *A. salmonicida* for example would greatly enhance our understanding of marine outbreaks of these diseases and role of jellyfish in stock loss. Perhaps with a little further research, cnidarian jellyfish can be added to the list of vectors of microbial disease for Atlantic salmon that already includes sea lice and wild fish (Barker et al., 2009; Belkin et al., 2005).

If the ability of jellyfish to transmit harmful microbes is confirmed, the true incidence of these bacteria within the microbiome must be explored. The resident microbes of cnidarians are known to vary with tissue site as well as species, location and even size of host (Brown et al., 2017; Weiland-Brauer et al., 2015). The isolation of microbes in this research may therefore be of little consequence to Atlantic salmon production, or they may represent core microbiota of the cnidarian microbiome. Further research utilizing a greater number of individuals and culture-independent methods such as 16S next generation sequencing would assist in answering this question. Existing research also suggests an involvement of the microbiota in venom production, through symbiosis with their jellyfish host. If this is the case, *A. salmonicida* and the *Vibrio* species isolated in this study might be considered potential candidates for this, due to their tissue erosive properties that might assist digestion of cnidarian prey.

A good deal of basic research must be conducted before conclusions can be drawn regarding the true impact of cnidarian organisms on fish health. Information on exposure density at which jellyfish, even microscopic species, can prove harmful is

essential. Perhaps a low density of cnidarians in the water column is of little consequence to farmed fish, but with increased bloom density or induction of stress within the population, disease may manifest. Or perhaps like low level phytoplankton exposure, cnidarian jellyfish also impact gill health and alter susceptibility to subsequent infectious disease. Little is understood about the mixed and complex aetiology of gill disease in farmed salmon, and much is still to be learnt about the potentially under-diagnosed impact of cnidarians and phytoplankton. With increasing reports of the influence of climate change on aquatic organisms, particularly on an altered marine environment that might favour cnidarian jellyfish (Mills, 2001; Purcell et al., 2007; Richardson et al., 2009), the understanding of the potential negative consequences of these organisms on farmed fish must be a priority for aquaculture research.

7.2 Microbial consequences of gill pathology

As has previously been discussed, altered immune status and tissue damage in gill tissue is associated with increased susceptibility to secondary infections. Results of Chapter 4 are suggestive of tissue damage and altered immune status due to phytoplankton exposure and existing research regarding the impact of jellyfish on salmonid health has demonstrated both gross and microscopically observable tissue trauma associated with exposure (M. D. Powell et al., 2018). Even without the vector transmission of microbes directly, these organisms negatively impact gill tissue, and might alter the bacterial communities therein. Many factors have been demonstrated to alter the microbiota of fish, particularly those that alter the tissue structure or immune function (Carding et al., 2015; Minniti et al., 2017; Reid et al., 2017). Dysbiosis or development of a disrupted microbiome with abnormal or pathogenic microbes (a pathobiome), is associated then with predisposition to further negative consequences, such as disease (Bass et al., 2019).

Publications regarding the human microbiome draw some fascinating conclusions regarding the alterations of microbial communities in different disease states. Altered microbiota has been demonstrated as a result of the onset and progression of cancer (Osman et al., 2018; Schwabe, 2013), and microbial changes have even been implicated

in induction of disease, with poorly understood conditions such as Crohn's having an apparent important multifactorial microbial component (Doherty et al., 2018; Pascal et al., 2017). Instead of a single causative agent, general trends in community composition appear important in the onset and severity of Crohn's disease. Bacteria are even used as indicators of recovery, or a poor prognosis (Salisbury et al., 2017; Shaw et al., 2019). The microbiome of gill tissue of farmed fish might therefore be of interest in monitoring fish health. There exist a number of studies interested in the microbiota of healthy fish; however few have looked at alterations to microbial communities with disease.

The work of the final chapters of this thesis was conducted with the aim of monitoring the health status of fish, in an attempt to observe shifts in microbial community composition with varied pathological states. Because fish are exposed to a multitude of factors in the marine environment, including many potentially harmful phytoplankton and cnidarians, microbial change was not investigated in association with a specific disease-causing agent. Rather, gill pathology was scored using histology to classify gills as none, mild, moderate or severely damaged. The microbial communities of these gills were then investigated and results assessed for differences in the microbiota between gill states.

The work of Chapter 4 and that of previous research in cnidarian-associated trauma in salmonids explored the impact of these organisms only in a controlled environment. However, the 'true' picture of exposure in the marine situation is infinitely more complex. Understanding the real-world impact of diseases is essential for monitoring and predicting the consequences of exposure of fish to harmful stimuli. Chapters 5 and 6 assess fish in a farmed situation to determine what impact organically occurring gill trauma of mixed cause might have on adherent microbial communities. With hindsight, this likely impeded the analysis of results. With so many confounding variables, date of sample collected ended up being the significant variable for differences in microbial community composition. Perhaps if a larger number of samples had been collected on a single occasion, with assessment of gill pathology and 16S next generation sequencing, significant differences might have been observed at the community level with varied pathology. As it was, it was not possible to observe a community-wide influence of gill

pathology on microbial community composition. Factors such as temperature, salinity and pH have all been demonstrated to alter microbial populations of fish, and likely fluctuated through the sampling regime, contributing more significantly to observed group differences and obscuring any impact of individual pathology. However at the genus level a number of statistically significant trends were observed with increased severity of gill pathology, suggesting the use of various taxa as indicator of gill health.

Fish treated with hydrogen peroxide demonstrated a clear difference in community composition before and after treatment, suggesting on-farm activities of aquaculture are may impact adherent populations. Despite the well-established ability of H_2O_2 to kill bacteria this study represents the first report of the impact of this chemical on the salmon microbiome. As a treatment frequently utilised in the production of fish, from hatcheries to the marine stage of production, a potentially negative impact of this chemical on the resident microbiota of gills might be of concern. Results suggest a clearance of gill adherent microbiota by H_2O_2 treatment, and variable recolonization with microbiota. Treatment appears to reduce specificity of community structure and introduce greater individual variation, potentially allowing the colonisation of gills with sub-optimal or even harmful microbes. This research also opens the door to many topics for future study, including the impact of other on-farm activities on microbial communities, such as the introduction of cleaner fish or thermolice treatments.

As discussed above, until environmental data regarding the exposure of fish to non-infectious harmful environmental organisms can be incorporated into this research, their association with gill disease cannot be determined. An incomplete sample set was collected by SSF employees during the 12-month period of this research that unfortunately could not be explored, however the samples remain archived and future work might attempt to associate the populations of these samples to results of gill pathology, or even as part of a longitudinal monitoring study at the study site. This information might allow for correlation of phytoplankton and cnidarian population levels with prevalent environmental conditions towards predictive models for these harmful organisms.

7.3 Monitoring gill health status in salmonids

7.3.1 Current Techniques

Current health monitoring in Atlantic salmon aquaculture involves gross and sometimes histological assessment of gill tissue on a routine basis. This gross assessment is used by producers and health professionals to quantify the level of gill pathology in sampled individuals using a simple scoring system, assisting in assumptions about the general health status of the stock. Gills without apparent clinical disease are scored as 0, with increasing score with increasing severity of pathology up to a high of 5. Gills are scored on two indices, appearance of generalized disease, and presence of amoeba-associated pathology (raised, grey-white mucoid plaques on the gill surface). The author is familiar with performing this gross scoring, and so it was utilized in addition to histological assessment of gill tissue used in Chapters 5 and 6 of this research. Based on the results of these two forms of assessment, gills broadly correlated in terms of gross and histological pathology. Histological scores were eventually used as an indication of gill disease in these chapters as a more quantifiable and less subjective means of determining and staging gill disease. Whilst gross assessment is a well-established method of assessing gill health, it does fail to identify various microscopically apparent changes to gills, particularly in mild, subtle disease, and scoring can be very subjective. In the cases where gross and histological assessment did not agree, a small number of fish were observed to have clinically apparent disease but were classified as having 'none' in the histological scoring system used. This is due to a flaw of the semi-quantitative scoring system, where a low numerical score (0-3) in observed histopathology is classified as 'no disease'. Some pathology is of course considered normal, however use of rigid numerical classifiers reduces the ability of informed assessment of this. Some gills were also observed to have histological disease without the appearance of gross pathology. This is to be expected, as some pathologies are less observable clinically (such as infection with epitheliocystis) or often too mild to be detected by visual assessment of gills alone (such as lamellar clubbing). Nonetheless, the majority of gross and histologically derived scores agreed. Gross scores are provided within appendix materials as additional metadata of the dataset, as well as in

contrast to histological results. Whilst no statistical analyses were performed for these, the apparent broad agreement of gross and histological results might be of reassurance to the aquaculture industry, where routine gross assessments are performed by farm personnel with much greater frequency than costly, destructive histological appraisal by specialists.

Taxonomic classification of bacteria is also a complex and error prone field of study, with the rigid definitions of genus, species and subspecies ill-suited for application to these prokaryotic organisms, given their capacity for rapid evolution and horizontal gene transfer. Designation of a bacterial species based on genomics is determined by quantitative similarities between the DNA of those bacteria, as well as differences to other species. This is may be a flawed concept when applied to bacteria due to the very nature of their evolution and transfer of their genomic material. It has utility however in identification of known pathogenic isolates by PCR.

7.3.2 Improving techniques

In order for an accurate assessment of fish health to be performed, appropriate techniques must be utilized, such as in sample collection and appraisal. The altered microbiota of starfish with staged wasting disease, for example, is assessed using punch biopsies for collection of the microbial tissue, due to the action in tissue decay and systemic nature of this pathology (Lloyd and Pespeni, 2018). It is well understood in human medicine that different sampling methods should be utilised for different applications in appraisal of microbial communities. For example, swabs are considered appropriate for assessment of the skin surface (Tedeschi et al., 2017), however biopsies are required for sampling the dermis (Prast-Nielsen et al., 2019). This level of information is not available for investigation of the microbiota of salmonids, where the majority of studies utilise swabbing for sample collection, likely due to ease of collection. Although studies exist regarding optimal sample storage and DNA extraction techniques of salmonid-derived samples, and the significant influence of these factors on results, there has been no comparison of sampling methodology. The work of Chapter 6, investigating the obtained microbial community composition from swab and

biopsy samples, provides important insight into the different indications for varied sampling methodology, and suggests that microbial community structure might vary based on their localization within gill tissue. Even these initial results are exciting as they suggest that, similar to existing observations in a multitude of other tissue types, the microbiota is specialized by compartment even within salmonid gills. For future research it is important to note too that not only do results vary in different regions of salmonid gills, results also vary depending on the sampling methodology. Experimental design and sample collection are key in research, for accurate results and valid conclusions. These results suggest varied indications for different sampling methods, and will hopefully inform experimental design of future researchers interested in microbial communities of gills.

Unlike previous studies associating varied microbial community structure with disease state, this study failed to observe significant community-wide differences with varied gill pathology. This remains an important area of study however, as the influence of the microbiota on the progression or even prognosis of disease continues to develop as a field of research. Further, laboratory-based challenge trials might investigate the influence of specific gill trauma in a controlled environment on the microbiota of gills without the multitude of compounding variables present in the marine environment. Investigating the progression and impact of dysbiosis in salmonid gills might yield exciting insight into the consequences of microbial disruption, and impaired health status of farmed fish. Study of pro and prebiotics in the treatment of salmonids for modulation of the GIT microbiota might also be applied in the future treatment of gills. Results of this research hint at variation in a small number of key microbiota with varied gill pathology, some of which might transpire to be biomarkers of health or even potential biotic treatments for improved immune function and disease resistance in farmed Atlantic salmon.

7.3.3 Prospective techniques

Investigation of the transcriptomic response to pathogens or gill insults is a well-established technique in the molecular research of salmonid health and physiology,

however its use is currently primarily within an academic or industry research setting. Some researchers are paving the road to commercial profiling of fish health using more advanced techniques, such as Xelect in St Andrews, a company that provides specialist genetics support to the aquaculture industry (https://xelect.co.uk/). Current research links AGD infection with increased Mucin 5 expression, a gene similarly detected as important in the gill response to *P. parvum*. Future industrial assays might include profiling of expression of this and other genes in health profiling of farmed fish, particularly in production of broodstock. The health status and disease resistance of these animals is of crucial importance to the aquaculture industry, with selection of optimal broodstock key to obtaining healthy and productive offspring. Restructuring of the microbiome is an expanding field of research in human medicine, currently informing on the incidence, progression and prognosis of many disease states. Microbiota have even been trialled as therapy in salmonids, with pre- and pro-biotics utilized to enhance the gut microbiome of various aquaculture species (Burr et al., 2005). Future work building on the results of this thesis might eventually assist in similar treatments of gill disease in fish. Combining histology with assessment of the microbiome for monitoring dysbiosis will be key to identifying microbial alterations with specific disease states. Notably, the apparent causative agent of AGD (*Neoparamoeba perurans*) is a unicellular organisms that feeds on bacteria, with induction of disease suggested as linked to specific bacterial taxa (Embar-Gopinath et al., 2005). Clearly the healthy microbiome, its components and alterations to in composition are then essential fields of research for understanding factors influencing disease in the aquaculture production of Atlantic salmon in particular.

Infectious agents can be controlled by vaccination or pharmaceutical therapy, however organisms such as phytoplankton and cnidarians are near ubiquitous components of the environment. Consequently, limiting the impact of these organisms presents an arguably more complex challenge. Nevertheless, their impact on fish health must still be assessed. Although infectious and harmful agents impact both wild and commercially reared fish, the focus of this research was the health of farmed salmonids, due in part to the disproportionate incidence of disease experienced by these fish, and also the availability of specimens. Whilst not all aspects of the impact of these harmful

environmental organisms and consequences of gill disease could be explored, results obtained are of interest in understanding the impact of these organisms on aquaculture production of commercially important salmonids. The study of the microbiome in particular is a fascinating and expanding field of research, although the majority of published literature in salmonids has so far focused on the communities of GIT tissue. The microbiota of the GIT is known to play an important role in the defence of the digestive tract, and it is proposed that the microbiota of the gills might also have important function in this role. Overall, results of this body of work present a number of interesting questions regarding the function and modulation of the gill adherent microbial community in farmed Atlantic salmon. Fish within the marine environment are known to experience a number of challenges to their immune system, many of which disproportionately impact gill tissue. No other essential organ within the fish is so exposed to the environment, and as such, fish gills are often found to be suffering a combination of pathogenic insults. Intensive production in aquaculture appears to exacerbate the impact of these conditions.

7.4 Final remarks

that the results of this thesis suggest that undiagnosed gill disease in salmonids might be partially attributable to exposure to harmful phytoplankton and cnidarian organisms in the marine environment. This research demonstrates the potential for these organisms to elicit harm in the commonly farmed species, Atlantic salmon and rainbow trout. These organisms were associated in this research with clear alterations to gill tissue, in the case of *P. parvum*, and potential additional alterations to tissue in the case of microbial transfer from cnidarian jellyfish. The microbial community of salmonid tissue is considered essential for the health and full functional capacity of fish and was considered an element of gill tissue that might be disrupted by these organisms. It was concluded that, before the impact of these organisms can truly be understood in the context of aquaculture production of salmonids, a great deal more work is required. Results investigating the microbiota of cnidarians certainly support previously reported hypothesis regarding cnidarian jellyfish as vectors of bacterial disease. The action of wild fish or even parasitic organisms such as sea lice as vectors of microbial disease is well established and considered in current biosecurity protocols of aquaculture production, but there is little effort to prevent exposure of fish to cnidarians. Despite the existing research documenting the impact on fish health from biofouling of net pens, and numerous recorded incidents of mass fish mortalities associated with bloom events, few producers consider the impact of cnidarian exposure within the marine environment. No datasets detailing seasonal cnidarian communities in coastal environments where fish farms are located exist, and as such, estimations of 'normal' baseline or potentially problematic cnidarian density are not possible. The results of this research suggest an additional avenue of gill disease from cnidarian jellyfish exposure. Further work must focus on understanding which species are of concern to salmonid production, and at what densities. Without routine water sampling and assessment of concurrent gill pathology, these aims cannot be achieved.

In addition, this study investigated the gill adherent microbiome, with the objective of identifying dysbiosis in microbial communities that may result in gill damage. Both phytoplankton and cnidarian jellyfish were considered to cause tissue disruption and

impaired immunity, however they are not the only cause. Gill disease in farmed fish is often complex, with specific causative agents impossible to determine due to concurrent infections of tissue. A laboratory-based study would have provided more conclusive evidence for the gill pathology and any subsequent microbial restructuring, although this would not have been representative of the actual experience of farmed fish. As opposed to a picture of microbial community structure resulting from specific trauma or infectious condition, results instead represent the microbiome of farmed Atlantic salmon over a one-year period, following induction to the marine environment. This work was conducted by sampling fish farmed in the marine environment; fish intended for eventual sale and consumption. Investigation of the gill health of these salmonids, exposed to varied non-infectious stimuli and infectious stimuli encountered in the marine stage of production, meant many avenues of microbial change that might result in infection. Results demonstrated a good deal of variation in the adherent microbial community, with influence of a number of described variables on community composition. Overall, results of this work indicate a less stable microbial community than previously assumed throughout the marine stage of Atlantic salmon production. On farm application of hydrogen peroxide appears particularly to impact microbial community composition, clearing many of the resident microbiota of the gills, resulting in increased individual variation and the potential for recolonization with harmful microbiota. Throughout the twelve-month period however community structure fluctuated. Although significant variation in a number of key microbes was detected, high individual variation could not be exclusively attributable to varied individual challenge, such as incidence or severity of disease. However, the influence of H_2O_2 on community composition was clear.

The results of Chapter 5 provide some evidence of disruption of adherent microbial communities associated with histologically observable gill disease. The overgrowth of harmful microbes linked to obvious disease is of course only one aspect of the potential consequences of this disruption. The microbiota of the GIT is well understood to perform important functions in many aspects of survival of fish, but also in the ability to thrive. Microbiome research in humans suggests involvement of these communities far beyond their host tissue location. Current salmonid microbial research is now exploring

the gill resident communities and might in future discover similar broad functionality of these microbes. If colonisation of gills by these microbes is inhibited by aquaculture production of fish (such as is suggested by studies contrasting farmed salmonids to wild counterparts (Webster et al., 2018)), there might be consequences for mutualistic relationships and health. Based on the results of this thes;'[l;is, not only can disruption occur with gill pathology that might be attributed in some cases to cnidarian and phytoplankton exposure, but that a multitude of variables impact microbial community composition. Diseases such as AGD and complex gill disease appear influenced by presence of microbiota, and existing gill disease or impaired immune function certainly exacerbate these conditions. Based on these results, multifactorial gill disease observed in the marine production of salmonids might reasonably be partially attributable to these organisms. Overall, gill homeostasis appears a delicate balance, with multiple compounding factors influencing the performance of this tissue, and occurrence of disease. Collectively, the work of this thesis enhances our understanding of the varied functions and responses of gill tissue, particularly as these relate to exposure of fish to harmful environmental organisms, cnidarian jellyfish and phytoplankton.

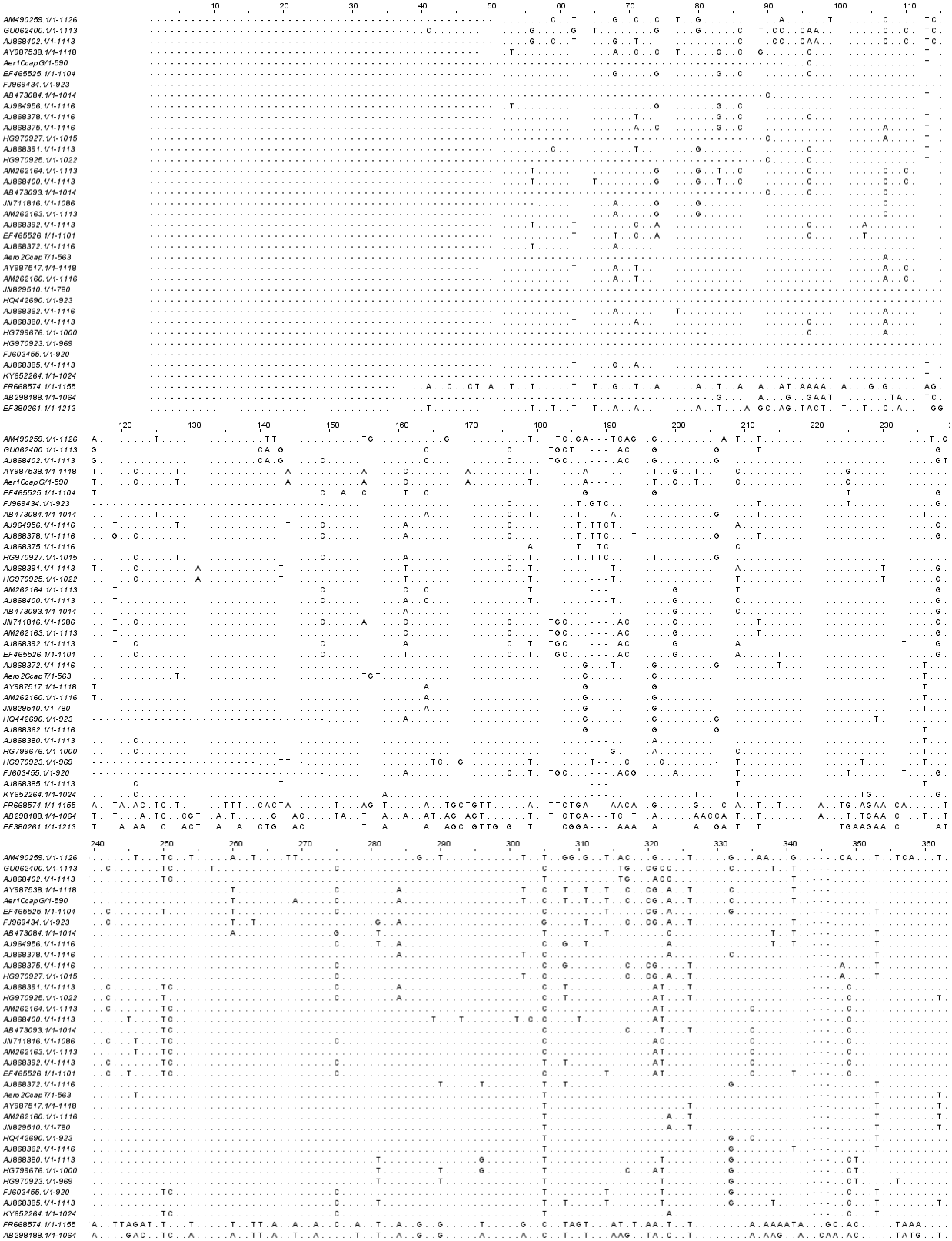
Appendix A

Appendix A; Sequencing data as part of the investigation of potential pathogens from the tissue of cnidarian jellyfish

Appendix A

A.1 Phylogenetic tree alignment (GyrB)

1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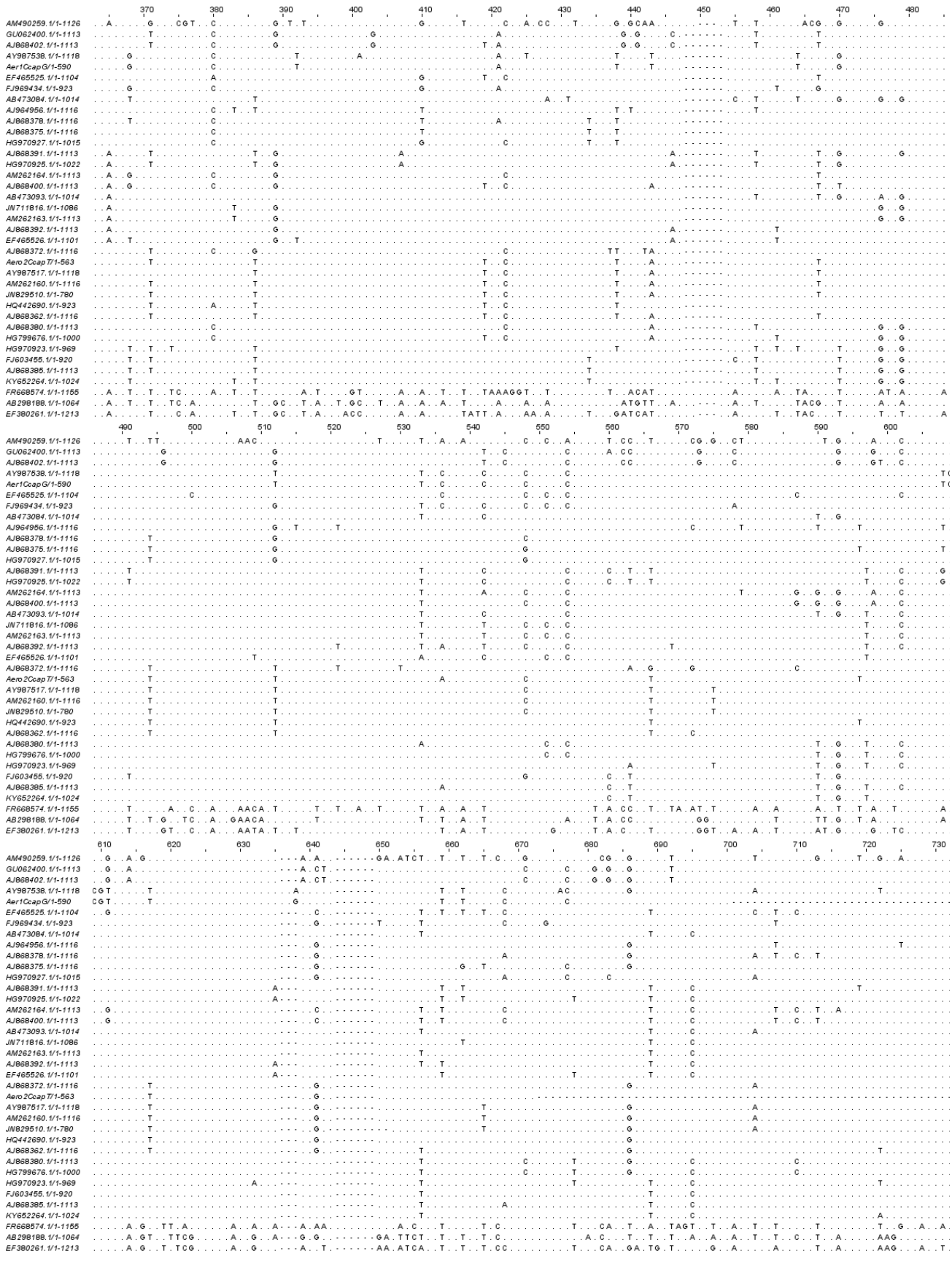
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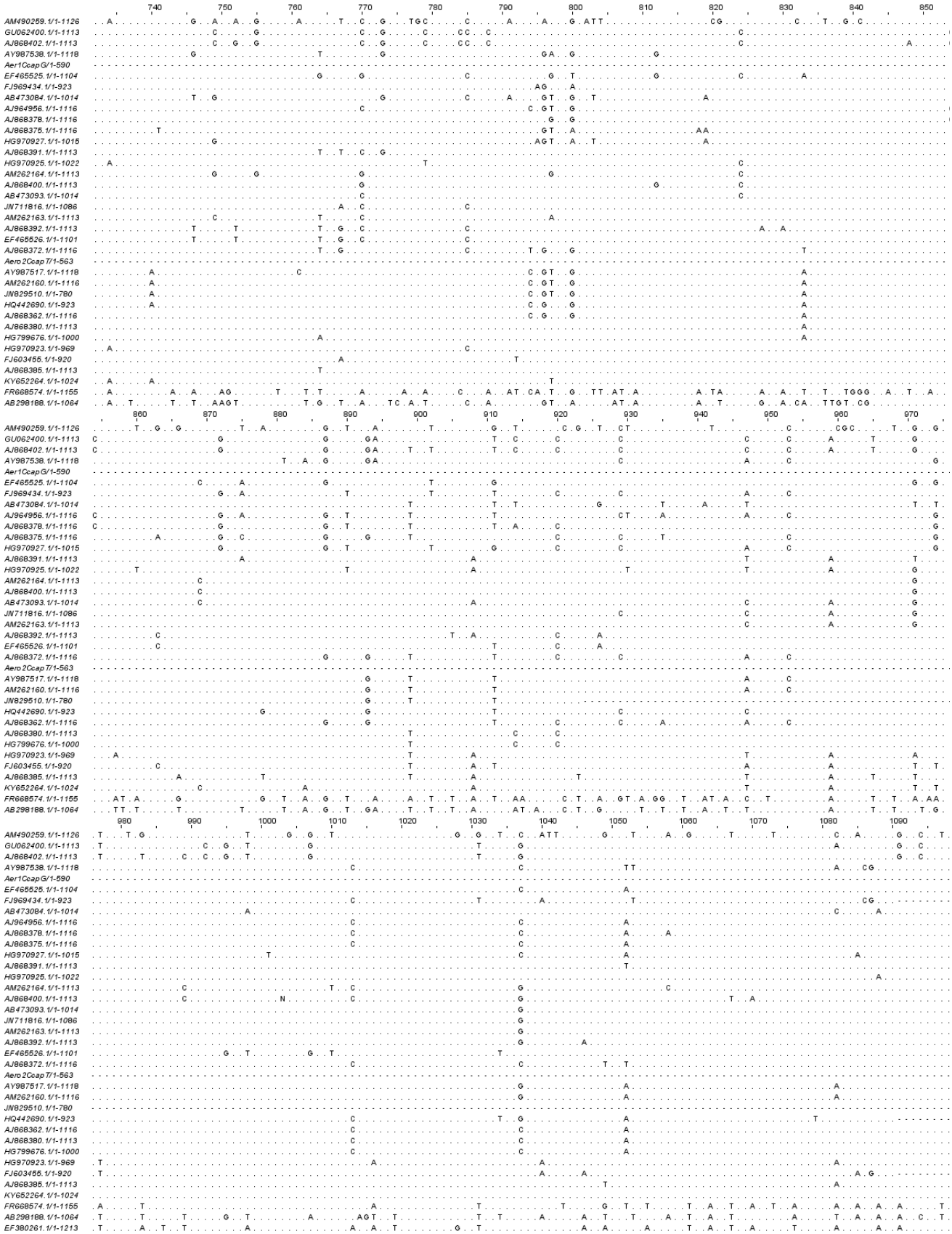
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FJ603455.1/1-920
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KY652264.1/1-1024
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980 990 1000 1010 1020 1030 1040 1050 1060 1070 1080 1090
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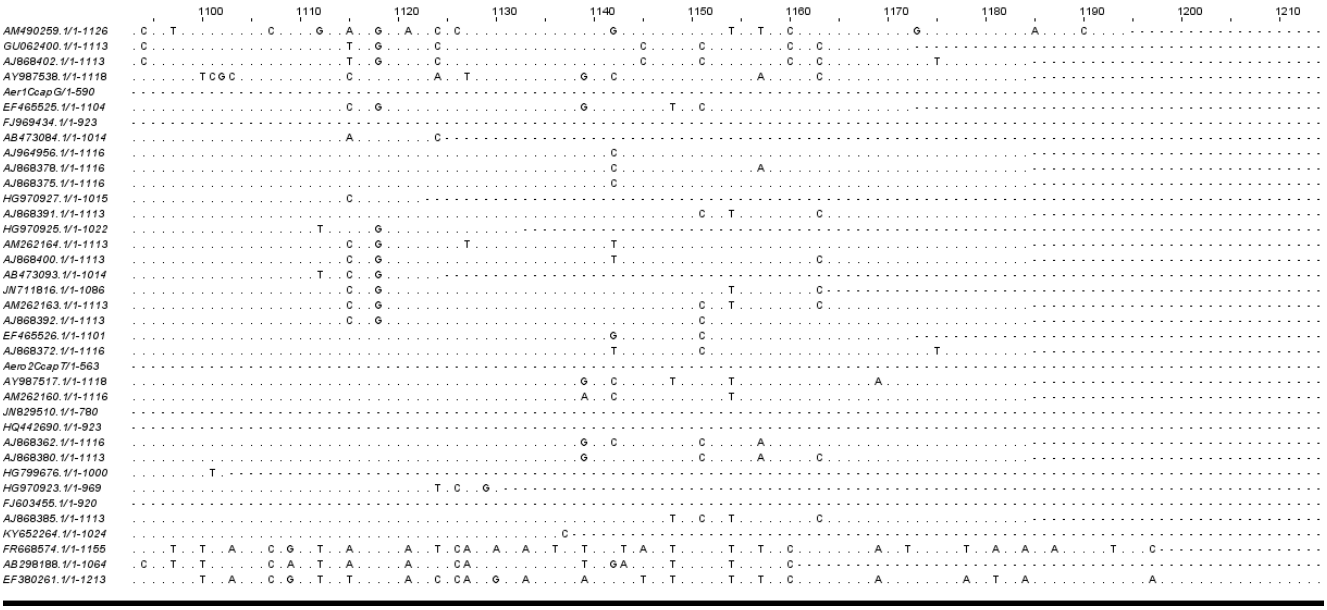
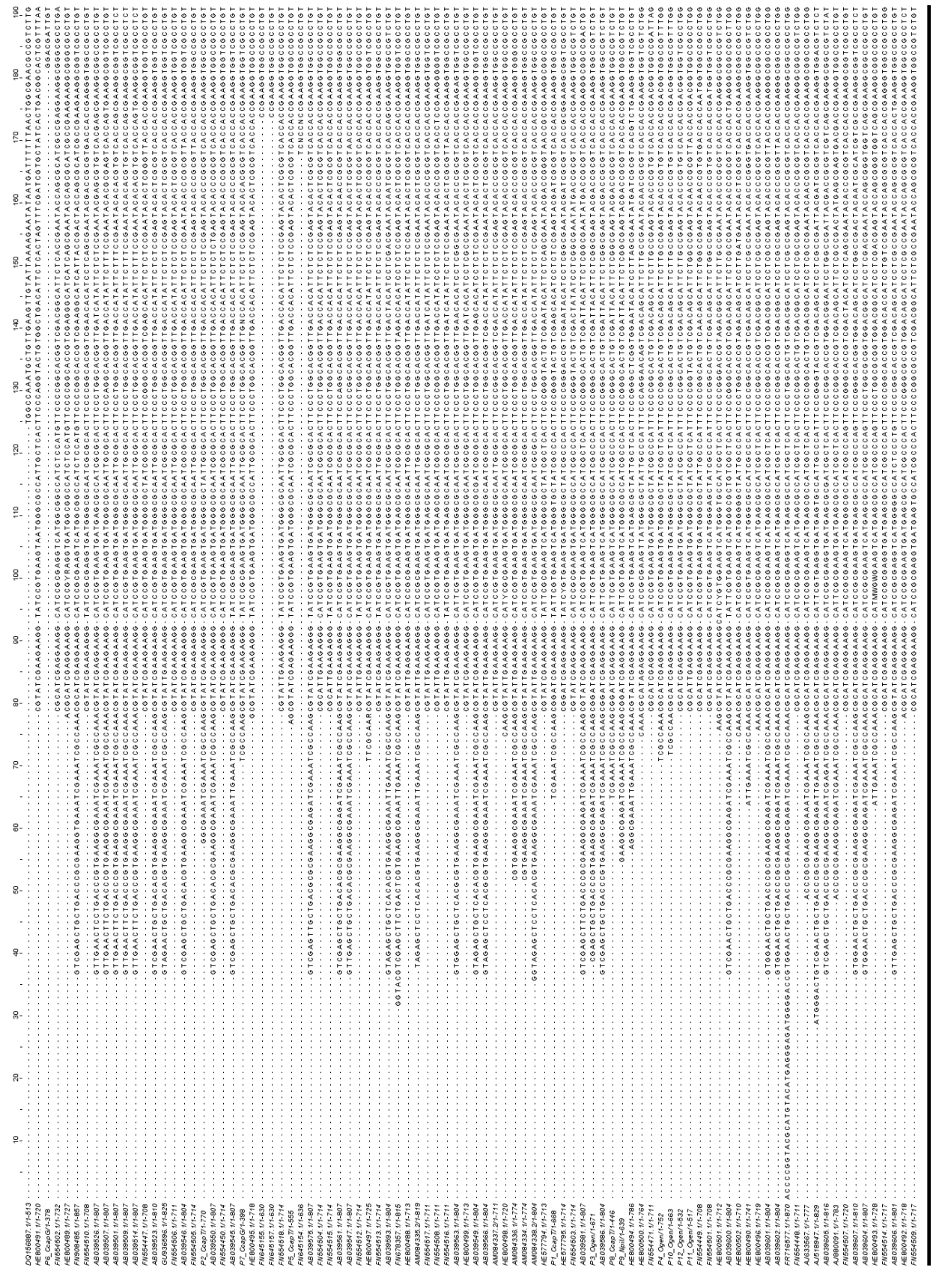
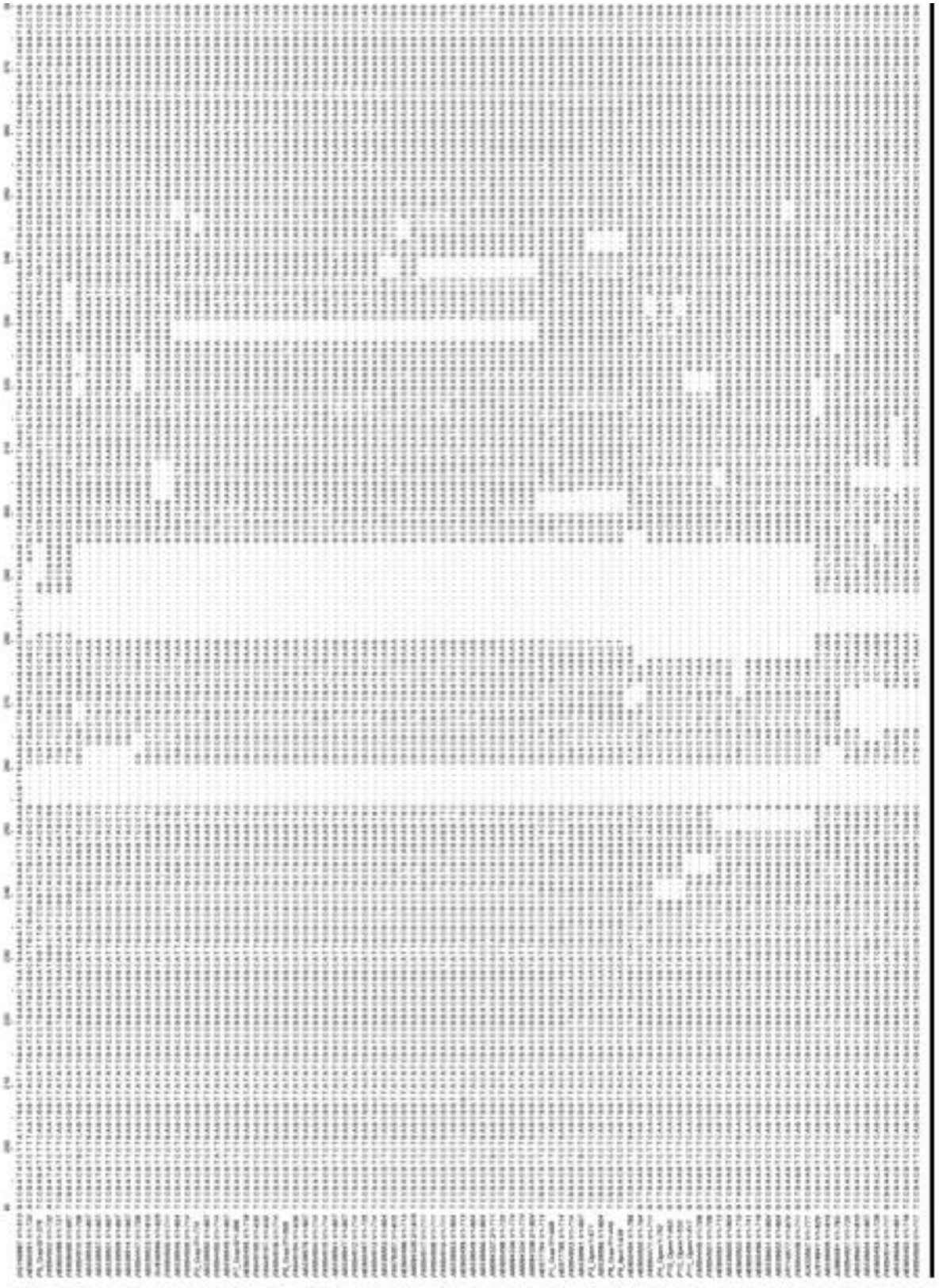


A.1: *Sequence alignment of Aeromonas GyrB gene sequencing results (Aero1CcapT and Aero2CcapG) and type strain sequences, for construction of phylogenetic tree (**Figure 3.2**). Type strains were obtained from Genbank database, based on curation status identified using the "stain info" resource. Alignment was generated using CLUSTAL default settings and constructed in Jalview. Identical nucleotide sequence is denoted by a dot (.) and regions without nucleotides are denoted with a dash (-). Regions of variation in sequence are illustrated with relevant nucleotides.*

A.2 Phylogenetic tree alignment (rpoD)





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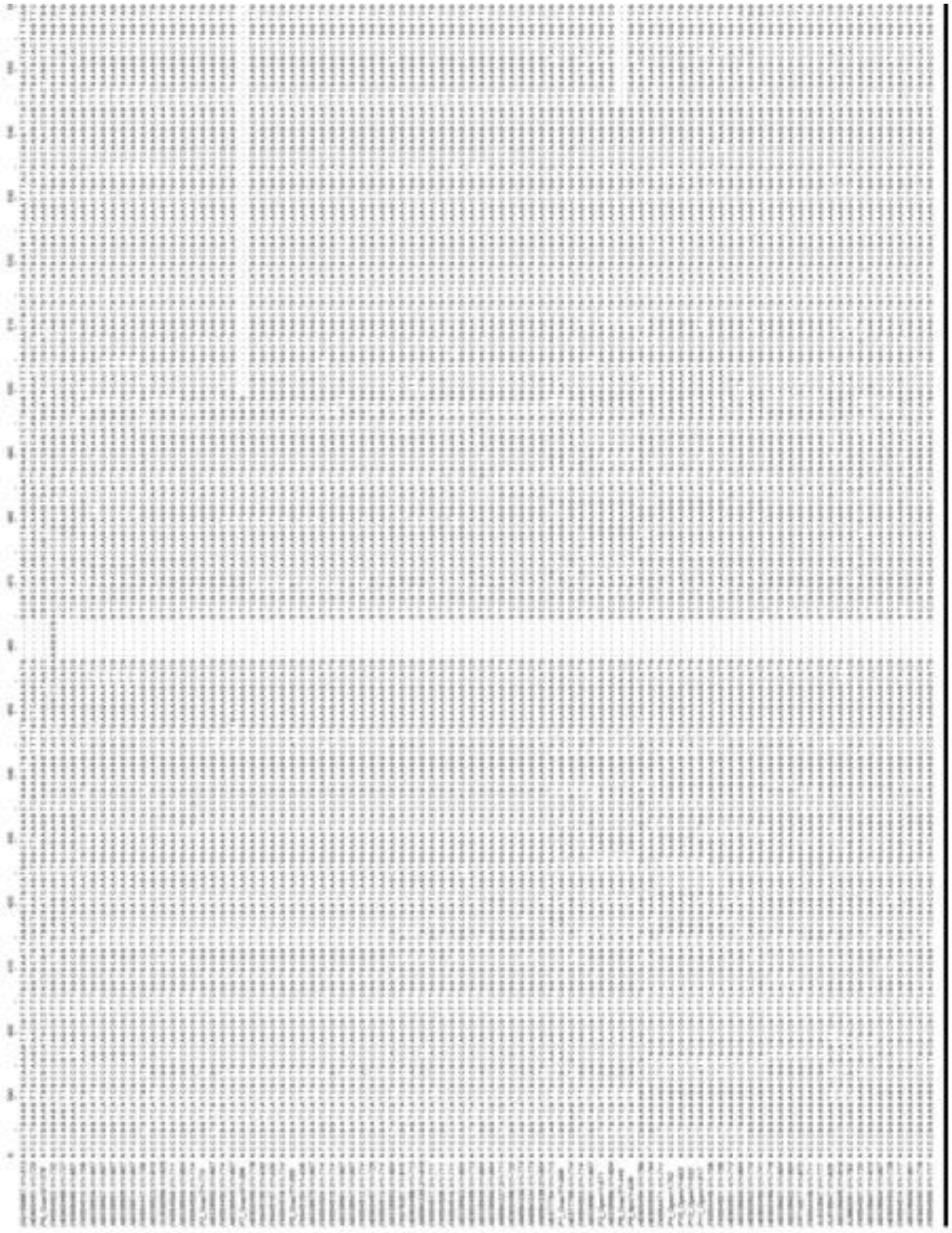

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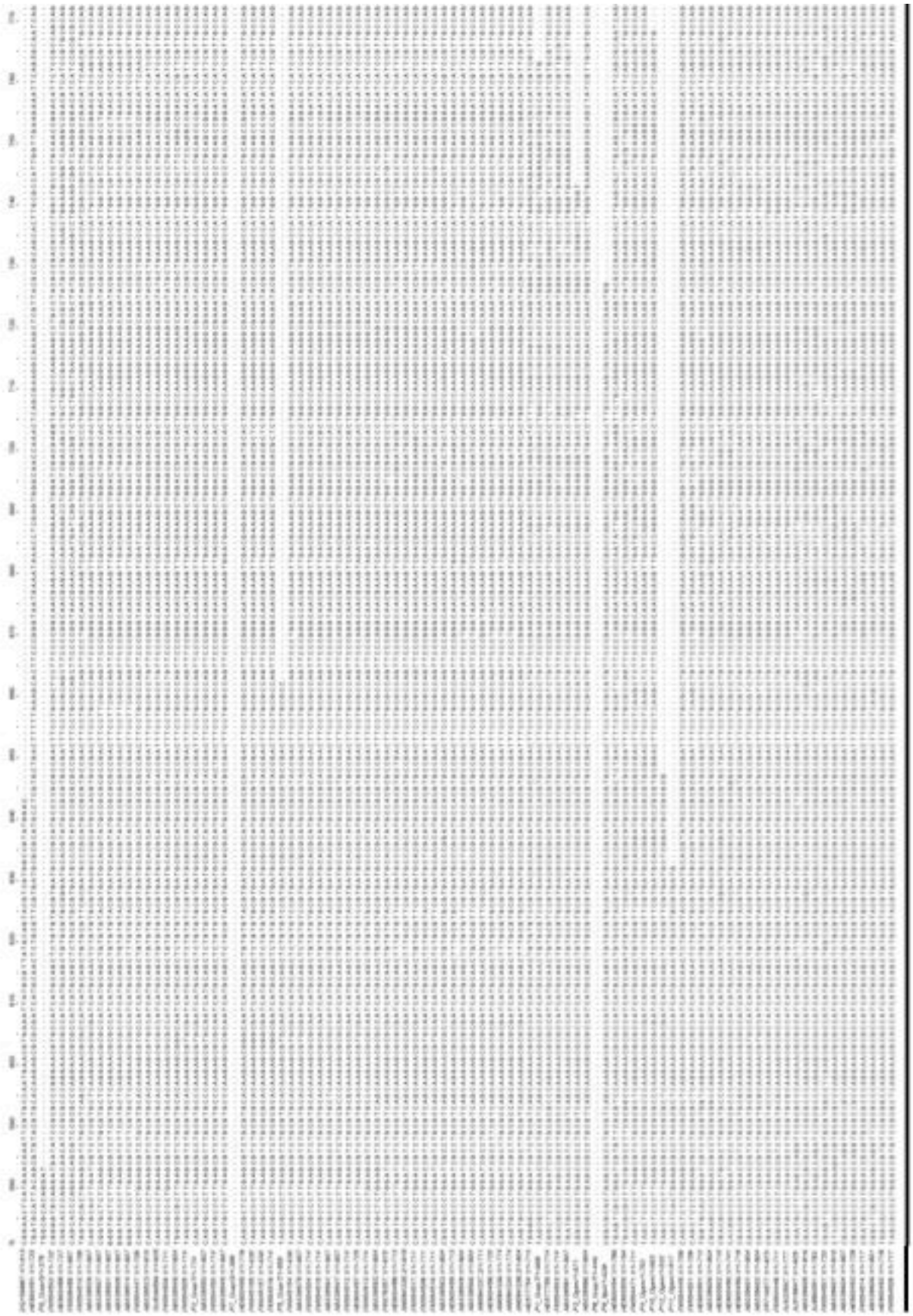

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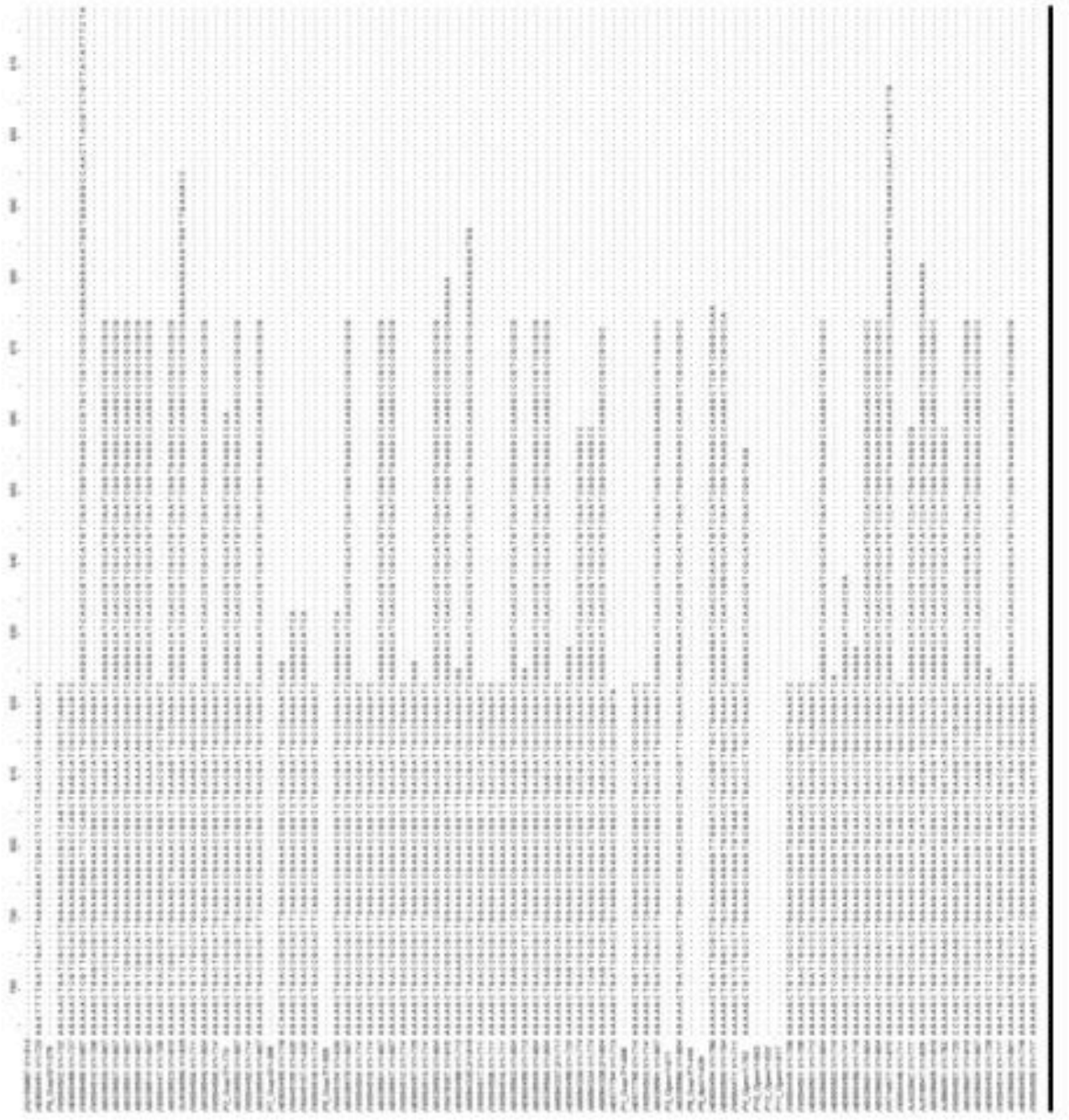
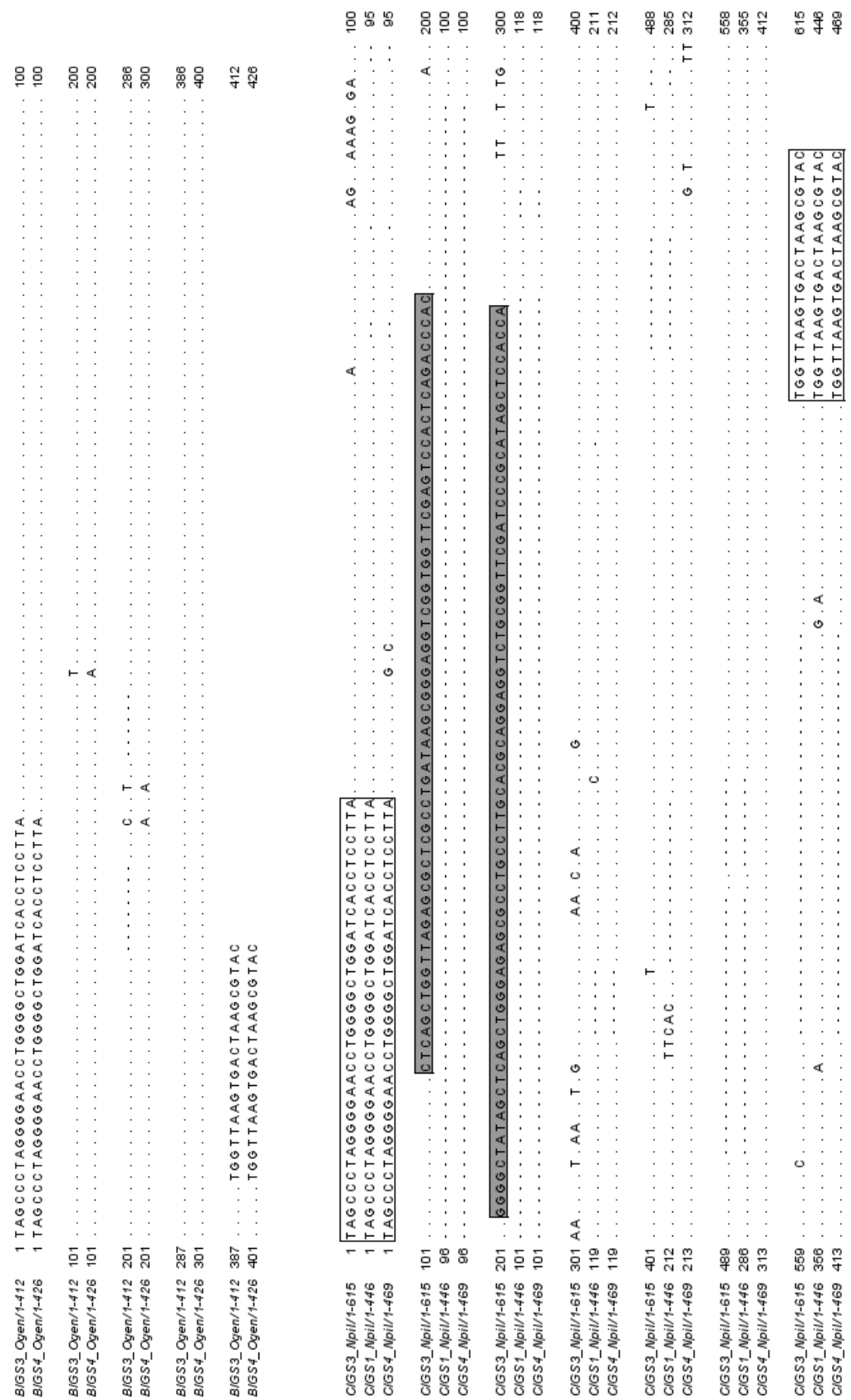


A.2: Sequence alignment of Pseudomonas rpoD gene sequencing results (P1 → P12) and type strain sequences, for construction of phylogenetic tree. Type strains were obtained from Genbank database. Alignment was generated using CLUSTAL default settings and constructed in Jalview. Regions without nucleotides are denoted with a dash (-)

A.3 Vibrio IGS alignments **B + C**



D

Appendix A

E + F

```
EIGS1_NpiI/1-461    1 TAGCCCTAGGGGAACCTGGGGCTGGATCACCTCCTTATACGAAGATACTTACGATGAGTGTCCACACAGATTGAT--GGTTTAG----------------  82
EIGS2_NpiI/1-631    1 TAGCCCTAGGGGAACCTGGGGCTGGATCACCTCCTTATACGAAGATACTCACGATGAGTGTCCACACAGATTGATTAGGTTTAGAAAGTAAAAGAGACGA 100

EIGS1_NpiI/1-461   83 --------------------------------------------------------------------------------------------------AT  84
EIGS2_NpiI/1-631  101 TATTGGGTCTGTAGCTCAGCTGGTTAGAGCGCTCGCCTGATAAGCGGGAGGTCGGTGGTTCGAGTCCACTCAGACCCACCAATATCGATTAGAAACAAAG 200

EIGS1_NpiI/1-461   85 TTAG--TTAAAGC-CAGAGCT--------------------------------------------------------------------------TTAAT 107
EIGS2_NpiI/1-631  201 ATGGGGCTATAGCTCAGCTGGGAGAGCGCCTGCCTTGCACGCAGGAGGTCTGCGGTTCGATCCCGCATAGCTCCACCATCTTTAAGGGTTTTTCCTTAAG 300

EIGS1_NpiI/1-461  108 TAATAACGTAAGTTATTGATTAAAGCTTTTTGCTTTATGCTCTTTAACAATTTGGAAAGCTGACTGATTAACTGACTTACGAGTCATGTTAATCAAATTT 207
EIGS2_NpiI/1-631  301 AATCTTTAAAAATGGTTTCGAAAGAAATCTAGCTCTTTAACAATTTGGAAAGCTGACTGATTGATTTACTTGCGAGTGAT-TCAATCAAATTTAAAAGTT 399

EIGS1_NpiI/1-461  208 AAAAGTTCTCAATGTTTATCTGCTCTTATTTAATAAGAACGGTATAAACACAACAAACACATTCAAGTGTCTTGTATTCGAATCAAATTTATTTGATTCA 307
EIGS2_NpiI/1-631  400 CTCAATGTTTATC--------TTTCATTAG------ATAAACACAACAAACACATTCAAGTGTCTTGTATTCGAATCAAATTTATTTGATTCACAATTGA 485

EIGS1_NpiI/1-461  308 CAATTGAGTCCGGCAAACAGTTATCAGGAATTAACCCTTCTTGATGACAACCAAAAACCTT----GGTTGCTTTTT-GTCTTCACTTTTTGAAAGTGAAA 402
EIGS2_NpiI/1-631  486 GTCCGGCAAACAGTTATCAGGAATTAACCCTTCTTGATGACAACCAAAAACCTTGGTTAGTTGCCATATGCTTATTTGTCTTCACTTTTT-AAAGTGAAA 584

EIGS1_NpiI/1-461  403 GCAAATTTGTATCATACACAAGACCTTTTCGGGTTGTATGGTTAAGTGACTAAGCGTAC                                         461
EIGS2_NpiI/1-631  585 GCAAAT------------AGAAACCCTTTCGGGTTGTATGGTTAAGTGACTAAGCGTAC                                         631
```

```
FIGS1_CcapG/1-426    1 TAGCCCTAGGGGAACCTGGGGCTGGATCACCTCCTTA..........T.................................................... 100
FIGS2_CcapG/1-411    1 TAGCCCTGGGGGAACCTGGGGCTGGATCACCTCCTTA..........C.................................................... 100
FIGS3_CcapG/1-371    1 ------------------------------------------------------..............................................  46

FIGS1_CcapG/1-426  101 .................................................................................................... 200
FIGS2_CcapG/1-411  101 ......................................................T............................................. 200
FIGS3_CcapG/1-371   47 .................................................................................................... 146

FIGS1_CcapG/1-426  201 .................................T..........................................C....................... 300
FIGS2_CcapG/1-411  201 .................................................................................................... 300
FIGS3_CcapG/1-371  147 ..A................................................................................................. 246

FIGS1_CcapG/1-426  301 ................................................G.................................G................. 400
FIGS2_CcapG/1-411  301 ...............................A.........................................N.......................... 400
FIGS3_CcapG/1-371  247 .................................................................................................... 346

FIGS1_CcapG/1-426  401 ....TGGTTAAGTGAACTAAGCGTAC                                                                           426
FIGS2_CcapG/1-411  401 ....TGGTTAA---------------                                                                           411
FIGS3_CcapG/1-371  347 ....TGGTTAAGTG-ACTAAGCGTAC                                                                           371
```

Appendix A

G + H

A.3: IGS sequence alignments for samples B → H confirmed as Vibrio. Multiple sequences obtained for each sample are aligned with concurrently obtained sequences to demonstrate within-sample variation in IGS nucleotide sequence. Alignments were performed using default settings of CLUSTER online alignment generator and constructed for visualisation using Jalview software. Regions of 16S and 18S nucleotide sequences are enclosed by boxes and tRNA encoding regions of IGS genes (as identified using online tRNA tool (http://lowelab.ucsc.edu/tRNAscan-SE/) are highlighted in grey. Identical nucleotide sequence is denoted by a dot (.) and regions without nucleotides are denoted with a dash (-). Regions of variation in sequence are illustrated with relevant nucleotides.

A.4 IGS BlastN match results

Sample	Sequence	Cover	Identity	E value	Match	Accession
AOgen	**AIGS1**	**100%**	**98%**	**0**	***Vibrio splendidus* LGP32 chromosome 1**	**FM954972.2**
		100%	79%	2.00E-63	*Vibrio tapetis subsp. tapetis* isolate *Vibrio tapetis* CECT4600 genome assembly, chromosome: A	LT960611.1
	AIGS3	**100%**	**98%**	**0**	***Vibrio splendidus* LGP32 chromosome 1**	**FM954972.2**
		100%	79%	2.00E-64	*Vibrio tapetis subsp. tapetis* isolate *Vibrio tapetis* CECT4600 genome assembly, chromosome: A	LT960611.1
	AIGS4	89%	96%	0	*Vibrio splendidus* strain ATCC 33125 16S-23S ribosomal RNA intergenic spacer, partial sequence	AF413024.1
		87%	92%	6.00E-134	*Vibrio splendidus* LGP32 chromosome 2	FM954973.2
	AIGS5	89%	96%	0	*Vibrio splendidus* strain ATCC 33125 16S-23S ribosomal RNA intergenic spacer, partial sequence	AF413024.1
		87%	92%	6.00E-134	*Vibrio splendidus* LGP32 chromosome 2	FM954973.2
	AIGS6	**100%**	**98%**	**0**	***Vibrio splendidus* LGP32 chromosome 1**	**FM954972.2**
		100%	92%	0	*Vibrio crassostreae* 9CS106 chromosome 1, complete sequence	CP016228.1
		100%	83%	1.00E-101	*Vibrio vulnificus* strain VV2014DJH chromosome 1, complete sequence	CP019320.1
		100%	80%	1.00E-101	*Vibrio vulnificus* strain 93U204 chromosome I, complete sequence	CP009261.1
		100%	79%	1.00E-97	*Vibrio vulnificus* strain FORC_037 chromosome I, complete sequence	CP016321.1
	AIGS7	**100%**	**98%**	**0**	***Vibrio splendidus* LGP32 chromosome 1**	**FM954972.2**
		100%	79%	2.00E-64	*Vibrio tapetis subsp. tapetis* isolate *Vibrio tapetis* CECT4600 genome assembly, chromosome: A	LT960611.1

	AIGS8	**100%**	**98%**	**0**	***Vibrio splendidus* LGP32 chromosome 1**	**FM954972.2**
		100%	79%	3.00E-61	*Vibrio tapetis subsp. tapetis* isolate *Vibrio tapetis* CECT4600 genome assembly, chromosome: A	LT960611.1
	AIGS9	100%	87%	2.00E-178	*Vibrio splendidus* LGP32 chromosome 1	FM954972.2
BOgen	**BIGS3**	**100%**	**99%**	**0**	***Vibrio splendidus* LGP32 chromosome 1**	**FM954972.2**
	BIGS4	100%	96%	0	*Vibrio splendidus* LGP32 chromosome 1	FM954972.2
CNpil	**CIGS1**	**100%**	**99%**	**0**	***Vibrio alginolyticus* strain K08M4 chromosome 1, complete sequence**	**CP017916.1**
		100%	96%	0	*Vibrio crassostreae* 9CS106 chromosome 1, complete sequence	CP016228.1
	CIGS3	100%	94%	0	*Vibrio splendidus* LGP32 chromosome 1	FM954972.2
		100%	92%	0	*Vibrio crassostreae* 9CS106 chromosome 1, complete sequence	CP016228.1
	CIGS4	84%	98%	0	*Vibrio splendidus* strain ATCC 33125 16S-23S ribosomal RNA intergenic spacer, partial sequence	AF413024.1
		79%	92%	4.00E-91	Uncultured *Shewanellaceae* bacterium clone I3K-356ITS 16S-23S ribosomal RNA intergenic spacer, partial sequence	AY933360.1
		79%	88%	2.00E-74	Uncultured *Pseudoalteromonas sp.* clone I50-488ITS 16S-23S ribosomal RNA intergenic spacer, partial sequence	AY932999.1
DNpil	**DIGS1**	**100%**	**97%**	**0**	***Vibrio splendidus* LGP32 chromosome 1**	**FM954972.2**
	DIGS2	**100%**	**98%**	**5.00E-158**	***Vibrio splendidus* LGP32 chromosome 1**	**FM954972.2**
		100%	90%	1.00E-108	*Vibrio crassostreae* 9CS106 chromosome 1, complete sequence	CP016228.1
		100%	85%	2.00E-83	*Vibrio splendidus* LGP32 chromosome 2	FM954973.2
	DIGS3	**100%**	**99%**	**0**	***Vibrio splendidus* LGP32 chromosome 1**	**FM954972.2**

	DIGS4	**100%**	**100%**	**1.00E-123**	***Vibrio splendidus* LGP32 chromosome 1**	**FM954972.2**
		100%	96%	2.00E-105	*Vibrio crassostreae* 9CS106 chromosome 1, complete sequence	CP016228.1
		100%	93%	2.00E-95	Uncultured *Shewanellaceae* bacterium clone I3K-356ITS 16S-23S ribosomal RNA intergenic spacer, partial sequence	AY933360.1
	DIGS5	**100%**	**99%**	**0.00E+00**	***Vibrio splendidus* LGP32 chromosome 1**	**FM954972.2**
	DIGS6	**100%**	**99%**	**0.00E+00**	***Vibrio splendidus* LGP32 chromosome 1**	**FM954972.2**
	DIGS7	**100%**	**99%**	**0.00E+00**	***Vibrio splendidus* LGP32 chromosome 1**	**FM954972.2**
ENpil	EIGS1	100%	93%	0	*Vibrio alginolyticus* strain K08M4 chromosome 1, complete sequence	CP017916.1
		100%	90%	1.00E-165	*Vibrio crassostreae* 9CS106 chromosome 1, complete sequence	CP016228.1
	EIGS2	100%	96%	0	*Vibrio alginolyticus* strain K08M4 chromosome 1, complete sequence	CP017916.1
		100%	93%	0	*Vibrio crassostreae* 9CS106 chromosome 1, complete sequence	CP016228.1
FCcapG	FIGS1	100%	95%	2.00E-177	*Vibrio splendidus* LGP32 chromosome 1	FM954972.2
	FIGS2	100%	95%	3.00E-156	*Vibrio splendidus* LGP32 chromosome 1	FM954972.2
		100%	93%	3.00E-146	Uncultured *Shewanellaceae* bacterium clone I3K-356ITS 16S-23S ribosomal RNA intergenic spacer, partial sequence	AY933360.1
	FIGS3	99%	95%	0	*Vibrio splendidus* LGP32 chromosome 1	FM954972.2
		92%	92%	5.00E-154	Uncultured *Shewanellaceae* bacterium clone I3K-356ITS 16S-23S ribosomal RNA intergenic spacer, partial sequence	AY933360.1
GOgen	**GIGS1**	**100%**	**98%**	**0**	***Vibrio crassostreae* 9CS106 chromosome 1, complete sequence**	**CP016228.1**

		100%	96%	0	*Vibrio alginolyticus* strain K08M4 chromosome 1, complete sequence	CP017916.1
	GIGS2	100%	99%	0	*Vibrio alginolyticus* strain K08M4 chromosome 1, complete sequence	CP017916.1
		100%	97%	0	*Vibrio crassostreae* 9CS106 chromosome 1, complete sequence	CP016228.1
	G_IGS3	100%	99%	0	*Vibrio alginolyticus* strain K08M4 chromosome 1, complete sequence	CP017916.1
		100%	98%	0	*Vibrio crassostreae* 9CS106 chromosome 1, complete sequence	CP016228.1
HCcapG	H_IGS1	100%	99%	0	*Vibrio splendidus* LGP32 chromosome 1	FM954972.2
		100%	97%	0	*Vibrio alginolyticus* strain K08M4 chromosome 1, complete sequence	CP017916.1
		100%	91%	1.00E-165	*Vibrio crassostreae* 9CS106 chromosome 1, complete sequence	CP016228.1
		100%	83%	7.00E-108	*Aliivibrio salmonicida* LFI1238 chromosome 1 complete genome	FM178379.1
		100%	82%	4.00E-100	*Vibrio fischeri* ES114 chromosome I, complete sequence	CP000020.2
		100%	82%	2.00E-94	*Vibrio fischeri* MJ11 chromosome I sequence	CP001139.1
	H_IGS2	**100%**	**99%**	**0.00E+00**	***Vibrio splendidus* LGP32 chromosome 1**	**FM954972.2**
	H_IGS3	**100%**	**99%**	**0**	***Vibrio splendidus* LGP32 chromosome 1**	**FM954972.2**

A.4: Results of comparison of obtained IGS sequences from Vibrio samples A – H against NCBI Genbank database by BlastN search. Positively identified sequences (based on the parameters of 100% coverage and 97% or higher identity) are highlighted in ***bold.*** *Results of consensus classification (when all positively identified sequences match via Genbank to the same species of Vibrio) are similarly in* ***bold****. Where multiple matches are considered significant, or matches do not meet the required criteria of positive identification, no consensus identification was obtained. Sufficient coverage was not obtained for sequences CIGS4 and FIGS3 (in grey).*

Appendix B

Appendix B: SDEGs, GO process and KEGG pathways results as part of the gill response to *P. parvum*.

B.1 Differential expression analysis of treatment groups relative to each other

Severe vs Mild	Positive fold Change
CP	412.43
CSF3	9.17
MRP63	8.26
USP2	7.62
DDIT4	6.88
C10orf118	6.17
SGK1	5.73
ZBTB1	5.55
DUSP2	5.47
ADAMTS1	5.19
SH3GL3	4.99
ANKRD9	4.98
CXCL2	4.69
C5orf54	4.68
CEP250	4.23
PIK3IP1	4.16
ACTC1	4.07
EGLN3	4.05
IL8	3.96
ARRDC3	3.79
PHOSPHO1	3.63
CRYAB	3.56
SMTN	3.54
TSC22D3	3.47
HSPA8	3.42
CYP1A1	3.36
HSPA1B	3.33
PEX10	3.32
PNP	3.31
THBS2	3.22
SUOX	3.21
PZP	3.18
SKA2	3.16
ZNF708	3.13
RGS5	3.07
ZNF259	3.04
HOXD3	3.01
THBS1	3.00
FOSL2	2.98
CITED2	2.98
IL1RAPL1	2.96
SLMO2	2.96
ARL5B	2.95
ZFP36L2	2.93
FOS	2.92
ETS2	2.92
CRY1	2.91
ERO1L	2.90
OLFM1	2.90
LAMA3	2.90
TOB1	2.89
SLC3A2	2.87
ITM2C	2.83
SGMS2	2.81
SOCS3	2.80
CDKN1B	2.80
PMP22	2.78
TFPI2	2.78
UGDH	2.77
IP6K2	2.75
LYVE1	2.74
RTL1	2.74
F3	2.73
CRISPLD2	2.72
TNXB	2.71
PIK3R5	2.70
PLAC8L1	2.69
GIMAP6	2.69
IL12RB	2.69
CCRN4L	2.64
HRNR	2.64
ERRFI1	2.62
RGS1	2.62

Severe vs Mild (cont.)	Positive fold Change
ZBED5	2.62
SOCS1	2.60
TMPRSS11B	2.58
FIBIN	2.57
CXCR4	2.57
TUSC2	2.56
PDE7A	2.53
TGM1	2.53
GLIPR2	2.51
ARG2	2.50
IRGC	2.50
C1orf170	2.49
HOXB3	2.48
KDELC2	2.48
LTB	2.47
PRSS23	2.44
GJA1	2.44
CCR9	2.43
IL11	2.42
ANGPTL4	2.41
BCL6	2.41
CAMK2N2	2.40
ANXA2	2.40
GADD45A	2.40
CASP3	2.39
BDNF	2.38
GRASP	2.38
IL1B	2.37
JUN	2.33
SOSTDC1	2.31
CTGF	2.30
PLEK2	2.29
ANGPTL2	2.29
ZFP36L1	2.29
ETS1	2.28
EIF1B	2.27
NUPR1	2.27
COQ10B	2.26
PFKFB3	2.26
RC3H2	2.25
SLC26A5	2.25
CRIP3	2.25
LAMC1	2.24
PIM3	2.20
HOXC13	2.20
CMKLR1	2.17
DVL2	2.17
PDK2	2.17
ZFAND5	2.15
ATP2B1	2.15
SSH2	2.14
IL1R2	2.14
SCN4A	2.12
CYR61	2.12
GLS	2.11
HMGB3	2.10
MEGF6	2.10
TP53INP2	2.08
PTGS2	2.08
TBC1D9	2.08
S1PR1	2.07
MKL1	2.07
ODC1	2.05
CDO1	2.04
EVA1B	2.04
PDXP	2.04
JUNB	2.04
GADD45B	2.03
NFKBIA	2.02
RASGEF1B	2.01
RASSF8	2.01
SCAND3	2.01
SNAI1	2.01

B

Severe vs Mild	Negative fold change
CHIA	-156.50
MUC5B	-50.67
ZBED5	-22.90
MTOR	-18.39
DMBT1	-7.45
MDH1	-6.97
GOLT1B	-5.38
ZG16	-5.01
RGS2	-4.23
MHCI	-4.01
MUC2	-3.71
PVALB	-3.38
LALBA	-3.22
PSG8	-3.16
HDDC3	-3.13
KPNA2	-3.07
GIMAP7	-3.04
CLEC17A	-3.03
AGR2	-2.82
CDCA7	-2.80
RAC2	-2.77
NRG4	-2.65
PNPLA4	-2.60
Nsg2	-2.60
FAM132A	-2.57
HVCN1	-2.55
GSTP1	-2.54
B3GNT9	-2.54
CRB3	-2.51
CCNB1	-2.48
UMPS	-2.47
UMOD	-2.47
ZNF771	-2.46
MEGF6	-2.44
ALOXE3	-2.41
GMNN	-2.40
STEAP4	-2.39
ORC4	-2.37
DLX1	-2.33
ID1	-2.24
GNA13	-2.23
B3GALT2	-2.21
CLDN4	-2.21
FLG2	-2.19
CCL4L2	-2.19
CIRH1A	-2.18
AHNAK2	-2.18
DTX3L	-2.17
ARL15	-2.15
TRIM16	-2.11
CSNK2A1	-2.10
CCL14	-2.09
SFT2D2	-2.05
MTFMT	-2.04
KRT17	-2.04
TST	-2.02

C

Severe vs Moderate	Positive fold change
PGBD4	10.659
THBS2	7.30559
IL1B	5.37401
IL1B	4.21637
IL1B	4.21344
IL1B	4.21344
LYVE1	2.85799
IL1B	2.58829
LTB	2.53327
SOCS3	2.36362
SOCS3	2.36362

D

Severe vs Moderate	Negative fold change
LMO7	-3.600007719
RALB	-2.703821666
GJA3	-2.127265346

B.1: Differential expression analysis of Severe relative to Mild group (A + B), as well as Severe and Moderate group (C + D) comparisons. SDEG represent those transcripts significantly altered (>2x fold change and <0.01 adj p) between different clinical presentation groups exposed to P. parvum. No SDEG were identified in a comparison of Moderate and Mild groups.

Multiple identical probes in microarray analysis assist in attenuating slide-specific effect, however, when probes are later assigned to a single HGNC gene, as above, results were then combined using the mean to prevent duplicates within gene lists. This has the potential to have omitted meaningful biological information from the study, through masking of outliers and loss of information regarding different isoforms. Future work might include further exploration of the nucleotide sequence of these transcripts, potentially yielding interesting information regarding their varied function.

Row Labels	Gene Count	BH adj. p
GO:0001558~regulation of cell growth	53	0.0094
GO:0001649~osteoblast differentiation	31	0.0092
GO:1903827~regulation of cellular protein localization	69	0.0088
GO:0042692~muscle cell differentiation	49	0.0086
GO:0007249~I-kappaB kinase/NF-kappaB signaling	38	0.0082
GO:0009306~protein secretion	60	0.0081
GO:0043588~skin development	36	0.0070
GO:0060537~muscle tissue development	50	0.0068
GO:0008544~epidermis development	43	0.0066
GO:0051348~negative regulation of transferase activity	49	0.0066
GO:0044772~mitotic cell cycle phase transition	65	0.0065
GO:0048598~embryonic morphogenesis	71	0.0056
hsa04630:Jak-STAT signaling pathway	30	0.0056
GO:0035295~tube development	72	0.0054
GO:0061061~muscle structure development	73	0.0053
GO:0040013~negative regulation of locomotion	39	0.0051
GO:0001822~kidney development	40	0.0051
GO:0042176~regulation of protein catabolic process	51	0.0050
hsa04110:Cell cycle	27	0.0047
GO:0043122~regulation of I-kappaB kinase/NF-kappaB signaling	37	0.0045
GO:0072001~renal system development	42	0.0044
GO:0098542~defense response to other organism	64	0.0044
GO:0055001~muscle cell development	27	0.0042
GO:0007162~negative regulation of cell adhesion	35	0.0042
GO:0000302~response to reactive oxygen species	34	0.0042
GO:0036294~cellular response to decreased oxygen levels	26	0.0032
GO:0009896~positive regulation of catabolic process	48	0.0031
GO:0051271~negative regulation of cellular component movement	40	0.0030
GO:0003007~heart morphogenesis	37	0.0026
GO:0031329~regulation of cellular catabolic process	53	0.0026
GO:0031099~regeneration	30	0.0023
GO:0045930~negative regulation of mitotic cell cycle	36	0.0022
GO:0000122~negative regulation of transcription from RNA polymerase II promoter	91	0.0020
GO:0045619~regulation of lymphocyte differentiation	27	0.0017
GO:0071456~cellular response to hypoxia	26	0.0017
GO:0035270~endocrine system development	26	0.0014
GO:0001890~placenta development	28	0.0013
GO:0048568~embryonic organ development	59	0.0011
GO:0001503~ossification	53	0.0009
GO:0007346~regulation of mitotic cell cycle	67	0.0009
GO:1903311~regulation of mRNA metabolic process	27	0.0008
GO:0033993~response to lipid	105	0.0006
GO:0001655~urogenital system development	50	0.0004
GO:1903708~positive regulation of hemopoiesis	32	0.0003
GO:0043009~chordate embryonic development	79	0.0002

Results of GO analysis for SDEG genes (relative to the control) within the Severe group dataset. Only those processes uniquely identified within the Severe group are illustrated (no significant enrichment within either Mild or Moderate groups)

B

Row Labels	Gene Count	BH adj. p
GO:0043524~negative regulation of neuron apoptotic process	20	0.00992523
GO:0016064~immunoglobulin mediated immune response	23	0.00992223
GO:0051702~interaction with symbiont	12	0.00987229
GO:0050878~regulation of body fluid levels	50	0.00972582
GO:0071417~cellular response to organonitrogen compound	48	0.0095626
GO:0050866~negative regulation of cell activation	22	0.00933852
GO:0019319~hexose biosynthetic process	14	0.00929988
GO:0051896~regulation of protein kinase B signaling	19	0.00892056
GO:0034329~cell junction assembly	25	0.00891864
GO:0060627~regulation of vesicle-mediated transport	46	0.00887011
hsa04660:T cell receptor signaling pathway	20	0.00882798
GO:0045620~negative regulation of lymphocyte differentiation	10	0.00858449
GO:0010468~regulation of gene expression	307	0.00851959
hsa05145:Toxoplasmosis	22	0.00841595
GO:0072332~intrinsic apoptotic signaling pathway by p53 class mediator	14	0.00834936
GO:1901566~organonitrogen compound biosynthetic process	113	0.00826537
GO:0007599~hemostasis	39	0.00813059
GO:1990266~neutrophil migration	16	0.00803746
GO:0008406~gonad development	27	0.00792333
GO:0071346~cellular response to interferon-gamma	20	0.0079052
GO:0030593~neutrophil chemotaxis	15	0.00790025
GO:1903317~regulation of protein maturation	15	0.00790025
GO:0032273~positive regulation of protein polymerization	18	0.00789595
GO:0070555~response to interleukin-1	18	0.00789595
GO:0051851~modification by host of symbiont morphology or physiology	12	0.00760048
GO:0002440~production of molecular mediator of immune response	23	0.00758394
GO:0006873~cellular ion homeostasis	57	0.00752116
GO:0002708~positive regulation of lymphocyte mediated immunity	14	0.00749652
GO:0045860~positive regulation of protein kinase activity	48	0.00738094
GO:0043043~peptide biosynthetic process	63	0.00724951
GO:0010467~gene expression	368	0.00723414
GO:0045596~negative regulation of cell differentiation	61	0.00719594
GO:0070613~regulation of protein processing	15	0.00715497
GO:0042113~B cell activation	30	0.00714082
GO:0034976~response to endoplasmic reticulum stress	32	0.00691827
GO:0019058~viral life cycle	47	0.0068286
GO:0032946~positive regulation of mononuclear cell proliferation	20	0.00680319
GO:0001906~cell killing	18	0.00665848
GO:0000165~MAPK cascade	76	0.00660376
GO:0050671~positive regulation of lymphocyte proliferation	20	0.00624456
GO:0042345~regulation of NF-kappaB import into nucleus	11	0.00613064
GO:0006309~apoptotic DNA fragmentation	7	0.00610241
GO:0072330~monocarboxylic acid biosynthetic process	25	0.00609303
GO:1901888~regulation of cell junction assembly	14	0.00599059
GO:0002695~negative regulation of leukocyte activation	21	0.00599011
GO:0044712~single-organism catabolic process	76	0.00594737
GO:0060284~regulation of cell development	77	0.00592961
GO:0033673~negative regulation of kinase activity	31	0.00584982
GO:0023014~signal transduction by protein phosphorylation	79	0.00567409
GO:0055082~cellular chemical homeostasis	67	0.00566584
GO:0040007~growth	83	0.00562663
GO:0002704~negative regulation of leukocyte mediated immunity	11	0.00525418
GO:0048468~cell development	155	0.00515075
GO:1904591~positive regulation of protein import	17	0.00503367
GO:0009615~response to virus	36	0.00503045
GO:0019216~regulation of lipid metabolic process	34	0.00502913
GO:0006518~peptide metabolic process	75	0.00501793
GO:0042306~regulation of protein import into nucleus	24	0.00500399
GO:0043604~amide biosynthetic process	69	0.00498384
GO:0002253~activation of immune response	55	0.00496351
GO:0046394~carboxylic acid biosynthetic process	32	0.00468253
GO:0050792~regulation of viral process	32	0.00468253

GO:0042108~positive regulation of cytokine biosynthetic process	13	0.00464315
GO:0002456~T cell mediated immunity	15	0.00462631
GO:0046427~positive regulation of JAK-STAT cascade	15	0.00462631
GO:1904894~positive regulation of STAT cascade	15	0.00462631
GO:0042102~positive regulation of T cell proliferation	17	0.00461246
GO:0051347~positive regulation of transferase activity	62	0.00460201
GO:0002285~lymphocyte activation involved in immune response	22	0.0045513
GO:0009409~response to cold	11	0.00452689
GO:0071347~cellular response to interleukin-1	16	0.00448009
GO:1902106~negative regulation of leukocyte differentiation	16	0.00448009
GO:0051172~negative regulation of nitrogen compound metabolic process	124	0.00447939
GO:0009725~response to hormone	78	0.00442898
GO:1904705~regulation of vascular smooth muscle cell proliferation	8	0.00439415
GO:1990874~vascular smooth muscle cell proliferation	8	0.00439415
GO:0042307~positive regulation of protein import into nucleus	17	0.00421175
GO:0032677~regulation of interleukin-8 production	13	0.00415117
GO:0051047~positive regulation of secretion	40	0.00411537
GO:0006919~activation of cysteine-type endopeptidase activity involved in apoptotic process	16	0.00406788
GO:0007044~cell-substrate junction assembly	16	0.00406788
GO:0046632~alpha-beta T cell differentiation	16	0.00406788
GO:2000379~positive regulation of reactive oxygen species metabolic process	16	0.00406788
GO:0033674~positive regulation of kinase activity	52	0.00405928
GO:0044248~cellular catabolic process	132	0.00388398
GO:0097194~execution phase of apoptosis	15	0.00372878
GO:0010634~positive regulation of epithelial cell migration	18	0.00350589
GO:0002685~regulation of leukocyte migration	23	0.00343034
GO:0033043~regulation of organelle organization	100	0.00340601
GO:1903532~positive regulation of secretion by cell	38	0.0033744
GO:0072606~interleukin-8 secretion	8	0.00332422
GO:0034504~protein localization to nucleus	41	0.00329763
GO:0010660~regulation of muscle cell apoptotic process	12	0.0032472
GO:0046631~alpha-beta T cell activation	19	0.00322332
GO:0007565~female pregnancy	26	0.00307985
GO:0048017~inositol lipid-mediated signaling	26	0.00307985
GO:0051897~positive regulation of protein kinase B signaling	16	0.0028305
GO:0051817~modification of morphology or physiology of other organism involved in symbiotic interaction	17	0.00273505
GO:0043408~regulation of MAPK cascade	66	0.00269069
GO:0051251~positive regulation of lymphocyte activation	35	0.00267557
GO:0048015~phosphatidylinositol-mediated signaling	26	0.00246753
GO:0032355~response to estradiol	20	0.00240487
GO:0010657~muscle cell apoptotic process	13	0.00226646
GO:0006417~regulation of translation	40	0.00216807
GO:0051048~negative regulation of secretion	27	0.00213405
GO:0022603~regulation of anatomical structure morphogenesis	94	0.00213367
GO:0051402~neuron apoptotic process	28	0.00213285
GO:0050778~positive regulation of immune response	67	0.00208857
GO:1903531~negative regulation of secretion by cell	25	0.00207941
GO:0014065~phosphatidylinositol 3-kinase signaling	18	0.00207802
GO:0007264~small GTPase mediated signal transduction	55	0.00200164
GO:0006006~glucose metabolic process	27	0.0018392
GO:1904019~epithelial cell apoptotic process	17	0.00171477
GO:0006897~endocytosis	66	0.00169326
GO:0051130~positive regulation of cellular component organization	104	0.00168836
GO:0034248~regulation of cellular amide metabolic process	43	0.00167793
GO:0050870~positive regulation of T cell activation	29	0.00151564
GO:1901699~cellular response to nitrogen compound	58	0.00149281
GO:0002696~positive regulation of leukocyte activation	38	0.00140378
GO:0051495~positive regulation of cytoskeleton organization	27	0.00138658
GO:1903524~positive regulation of blood circulation	17	0.00138564
GO:0045862~positive regulation of proteolysis	42	0.00138509
GO:1901701~cellular response to oxygen-containing compound	85	0.00138252
GO:0043603~cellular amide metabolic process	91	0.00137265
GO:2001237~negative regulation of extrinsic apoptotic signaling pathway	19	0.00129507
GO:1903039~positive regulation of leukocyte cell-cell adhesion	30	0.00124262

GO:0050867~positive regulation of cell activation	39	0.001215
GO:0002712~regulation of B cell mediated immunity	12	0.00114597
GO:0002889~regulation of immunoglobulin mediated immune response	12	0.00114597
GO:0044255~cellular lipid metabolic process	95	0.00114035
GO:0032535~regulation of cellular component size	42	0.00112574
GO:0032844~regulation of homeostatic process	50	0.00111647
GO:0044706~multi-multicellular organism process	30	0.00099026
GO:0006629~lipid metabolic process	116	0.00096292
GO:0043523~regulation of neuron apoptotic process	28	0.00096115
GO:0060341~regulation of cellular localization	80	0.00095937
GO:1901214~regulation of neuron death	34	0.00085039
GO:0071214~cellular response to abiotic stimulus	35	0.00083648
GO:0002673~regulation of acute inflammatory response	16	0.00078215
GO:0002698~negative regulation of immune effector process	19	0.0007177
GO:0006970~response to osmotic stress	15	0.00071564
GO:0002449~lymphocyte mediated immunity	37	0.00056443
GO:0048878~chemical homeostasis	97	0.00056041
GO:0071260~cellular response to mechanical stimulus	16	0.00050207
GO:0070372~regulation of ERK1 and ERK2 cascade	34	0.00047736
GO:0010563~negative regulation of phosphorus metabolic process	59	0.00045392
GO:0045936~negative regulation of phosphate metabolic process	59	0.00045392
GO:0002250~adaptive immune response	49	0.00041859
GO:0050777~negative regulation of immune response	22	0.00040372
GO:0016192~vesicle-mediated transport	130	0.00038212
GO:0044085~cellular component biogenesis	227	0.00037094
GO:0010562~positive regulation of phosphorus metabolic process	98	0.00036346
GO:0045937~positive regulation of phosphate metabolic process	98	0.00036346
GO:0009719~response to endogenous stimulus	134	0.00035572
GO:0070997~neuron death	37	0.00034073
GO:0044092~negative regulation of molecular function	102	0.00032416
GO:0070371~ERK1 and ERK2 cascade	34	0.00031155
GO:0032615~interleukin-12 production	14	0.00029597
GO:0032655~regulation of interleukin-12 production	14	0.00024205
GO:0072331~signal transduction by p53 class mediator	27	0.00022786
GO:0032637~interleukin-8 production	16	0.00022675
GO:0006605~protein targeting	73	0.00018778
GO:0032787~monocarboxylic acid metabolic process	62	0.00016165
GO:0051345~positive regulation of hydrolase activity	90	0.00015811
GO:0051170~nuclear import	40	0.00015547
GO:0001934~positive regulation of protein phosphorylation	88	0.0001545
GO:0090066~regulation of anatomical structure size	56	0.00014794
GO:0032943~mononuclear cell proliferation	37	0.00014502
GO:0050776~regulation of immune response	90	0.0001317
GO:0045859~regulation of protein kinase activity	76	0.00012833
GO:0046651~lymphocyte proliferation	37	0.00012455
GO:0051128~regulation of cellular component organization	191	0.00011051
GO:0031401~positive regulation of protein modification process	108	9.3501E-05
GO:1902593~single-organism nuclear import	39	9.3415E-05
GO:0006606~protein import into nucleus	39	8.8575E-05
GO:0044744~protein targeting to nucleus	39	8.8575E-05
GO:0045087~innate immune response	85	8.7208E-05
GO:0045184~establishment of protein localization	171	8.5727E-05
GO:0008104~protein localization	201	6.7939E-05
GO:0042327~positive regulation of phosphorylation	93	6.3287E-05
GO:0019752~carboxylic acid metabolic process	85	5.9073E-05
GO:2001236~regulation of extrinsic apoptotic signaling pathway	28	5.3738E-05
GO:0042592~homeostatic process	146	4.9404E-05
GO:0070661~leukocyte proliferation	40	3.9962E-05
GO:0043085~positive regulation of catalytic activity	141	2.891E-05
GO:0051338~regulation of transferase activity	97	1.9749E-05
GO:0043549~regulation of kinase activity	84	1.9416E-05
GO:0006468~protein phosphorylation	164	1.2151E-05
GO:0008637~apoptotic mitochondrial changes	25	8.323E-06
GO:0019220~regulation of phosphate metabolic process	149	4.2339E-06
GO:0044093~positive regulation of molecular function	166	3.6335E-06

GO:0044093~positive regulation of molecular function	166	3.6335E-06
GO:0051174~regulation of phosphorus metabolic process	150	2.8248E-06
GO:0051336~regulation of hydrolase activity	130	2.4459E-06
GO:0006793~phosphorus metabolic process	254	2.149E-06
GO:0006796~phosphate-containing compound metabolic process	254	1.9206E-06
GO:0031399~regulation of protein modification process	155	1.812E-06
GO:0001932~regulation of protein phosphorylation	128	1.7906E-06
GO:0048585~negative regulation of response to stimulus	135	7.1231E-07
GO:0016310~phosphorylation	198	5.8532E-07
GO:0050790~regulation of catalytic activity	218	4.9398E-09

C

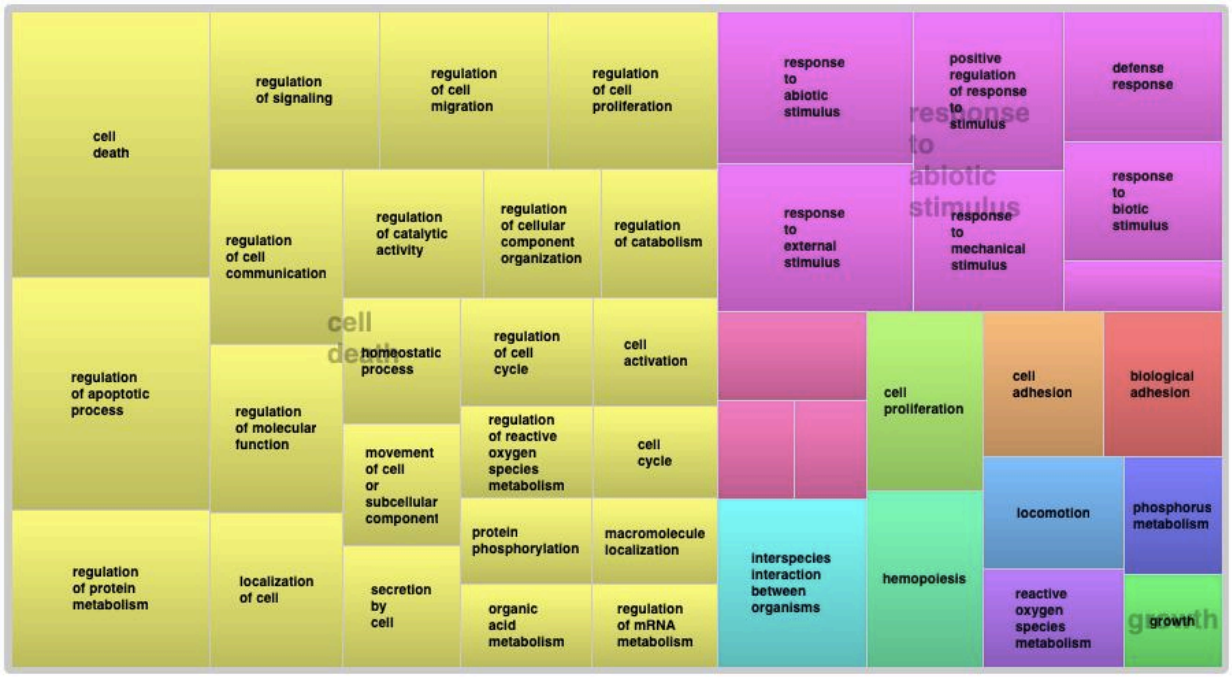
Row Labels	Gene count	BH adj. p
GO:0032635~interleukin-6 production	13	0.0065277
GO:0042254~ribosome biogenesis	25	0.00669946
GO:0045428~regulation of nitric oxide biosynthetic process	9	0.00997011
GO:0061041~regulation of wound healing	14	0.00756934
GO:1903034~regulation of response to wounding	16	0.00331827
GO:1903426~regulation of reactive oxygen species biosynthetic process	10	0.00994321
hsa05140:Leishmaniasis	12	0.00864141
hsa05152:Tuberculosis	21	0.00314477

Results of GO enrichment analysis for Severe (A), Moderate (B) and Mild (C) SDEG relative to the control. Only those pathways unique to each clinical presentation group are illustrated (not identified as enriched within other clinical presentation group, based on the parameters of a p value <0.01)

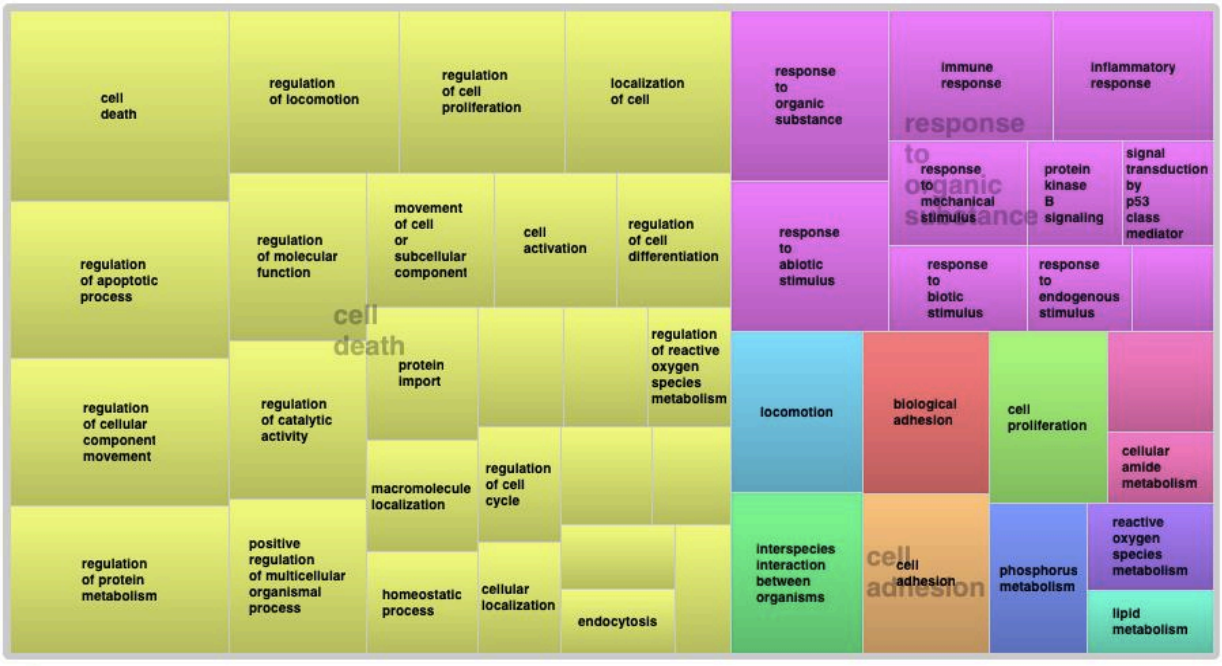
Only data for 'gene count' and DAVID generated significance values (Benjamini adjusted p values) have been included as these represent the meaningful information obtained from the DAVID database. Adjusted p values are useful for comparison within a dataset but not across gene lists due to the variable number of genes within those lists. Total gene count of SDEG involved in a pathway is therefore the most biologically meaningful value, however care must be taken in interpretation, as less specific GO terms tend to be composed of more genes. Example: A 100 gene involvement in 'Metabolic process' is less biologically interested than 10 genes involved in 'regulation of reactive oxygen species biosynthetic process'. Less is therefore not always more in the context of GO term analysis.

B.3 REVIGO visualisation

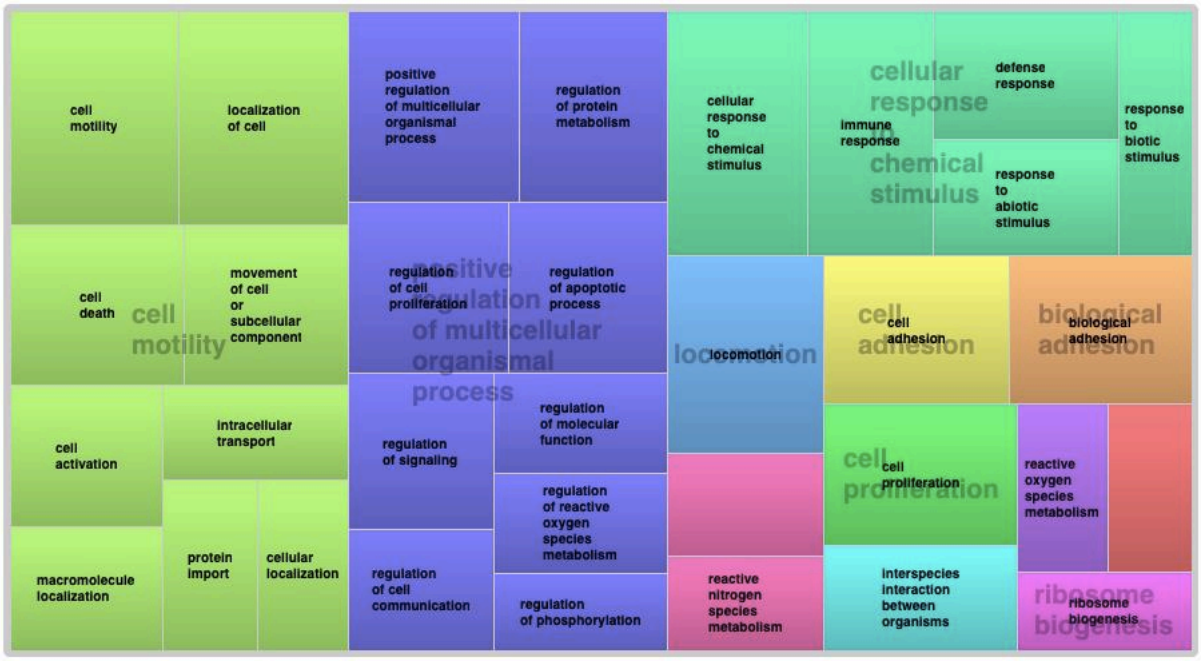
Severe



Moderate



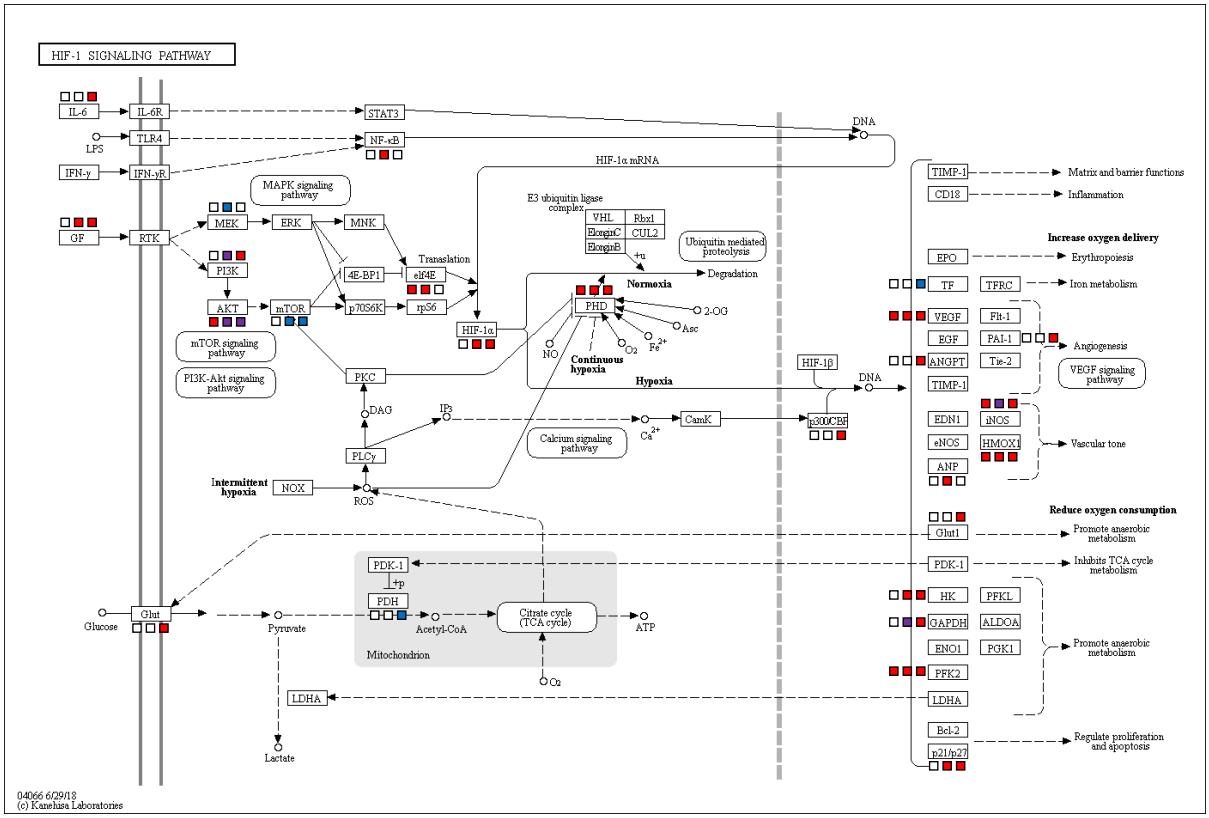
Mild



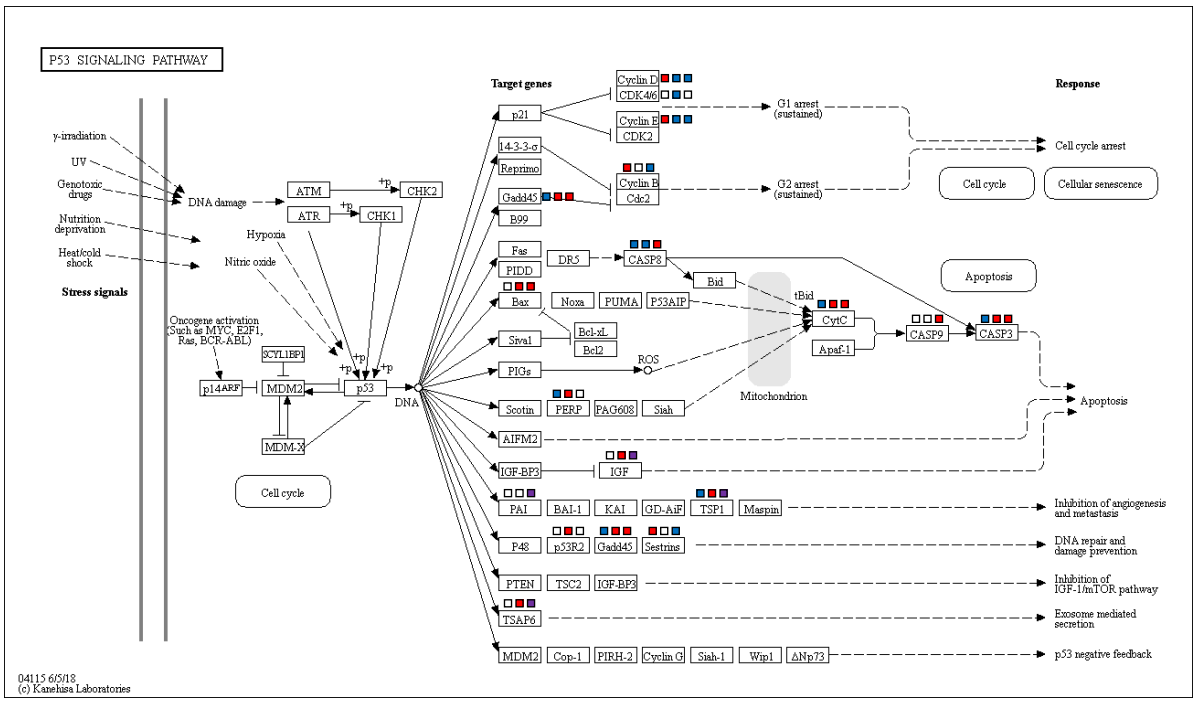
B.3: Visualisation of collapsed-term GO processed (where individual GO terms are collated into broader functional categories by online tool, REVIGO http://revigo.irb.hr/ using default website settings). Similarity can be observed in activity of Severe and Moderate group biological processes, with dominance of cell death processes compared to lower concentration treated Mild group.

B.4 KEGG pathways

HIF-1a signalling



P53 signalling



B.5 Select SDEG genes

HGNC symbol	Severe	Moderate	Mild
ABCB11	3.01		
ABCB4	-3.57		
ABCB8	-2.13		
ABCC4	3.01		-2.33
ABCF2	3.63	5.01	4.01
ABCF3	2.26	2.41	2.72
ABCG2	-5.86	-7.05	-3.07
ALDOA	2.53		
AMBP	10.18		
AMP	6.81	9.13	8.45
AMPD1	3.12	2.82	
ANGPT2	0.00	0.00	2.11
ANGPTL4	-1.17	0.00	0.00
ANGPTL4	-1.17	0.00	0.00
ANPEP	0.00	0.00	-0.55
APOA2	6.98	-3.65	
APOA4	-2.94		
APOO	8.53	7.51	5.61
AQP4	5.84		7.08
ARF1	4.99	2.12	
ARFRP1	-2.00		
ARHGAP24	0.00	0.00	3.23
ARL15	-4.07	-2.88	
ARL2BP	-2.17		
ARL4A		-2.61	-2.52
ARL4C	4.14	2.76	2.50
ARL5B	6.11	3.40	2.07
ARL9	-2.27	-2.22	
ATP1A1	2.87		
ATP1A3	3.04	2.70	3.27
ATP1B1	5.43	2.45	2.22
ATP2A1	2.90	2.38	2.11
ATP2A2	-2.32	5.85	2.09
ATP2B1	4.42	3.10	2.68
ATP5A1	4.03		
ATP5G3	2.22	2.72	2.09
ATP6V0E1	2.52		
BAX	0.00	1.00	0.00
BBS5	-2.23	-2.02	
BTG1	0.00	0.00	1.24
C1GALT1	0.00	0.00	1.18
C1QTNF2	2.05		
C1QTNF3	4.07		
C3	11.03	62.97	2.76
C3	-9.49	-4.01	-4.68
C6orf165	-8.61		
CASP10	3.31	2.40	2.21
CASP3	5.50	2.95	2.30
CASP8	3.16	2.02	2.03
CASP9	2.96		
CAT	-2.23		
CD1C	8.42	8.71	6.90
CD2	-2.02		
CD200	2.11	2.13	2.01
CD200	-2.27	-2.63	2.01
CD22	2.30	2.46	2.34
CD248	2.03		
CD274	2.22	2.35	
CD28	2.07	2.12	
CD300LF	-3.91		
CD40	3.11	2.29	
CD46	2.02		
CD55	5.35	4.00	7.01
CD68	-2.80		
CD83	2.31	4.11	

HGNC symbol	Severe	Moderate	Mild
CD300LF	-3.91		
CD40	3.11	2.29	
CD46	2.02		
CD55	5.35	4.00	7.01
CD68	-2.80		
CD83	2.31	4.11	
CD84		-4.01	
CD99	4.79		
CDKN1B	3.05		
CDKN1C	2.67		
CEACAM1	0.00	1.86	0.00
CES1	2.87		
CITED2	2.60		
CLEC11A	2.63		
CLEC17A	-2.52		
CLEC4E	2.31	2.40	2.49
CLIC2	2.85	2.06	
CLIC4	-2.65	-2.26	
COX6B1	7.80		
COX7C	-2.92		
COX8A	13.69		
CP	398.66		
CTLA4	2.52	2.55	
CXCL10	-4.49	-3.28	-3.83
CXCL12	2.06		
CXCL14		-2.45	-2.35
CXCL2	23.92	10.08	5.27
CXCR4	3.42		
CYP1A1	3.56	0.00	1.81
CYP27A1	-4.59	-4.50	
CYP2F1	-2.39	-2.18	
CYP2J2	-2.08	-2.28	
DDIT4	8.04	2.90	
DDIT4L	2.68	2.83	2.61
GADD45A	3.18	3.88	2.75
GADD45B	2.34		
GPI	0.00	-2.14	0.00
GPX2	-2.16		
GSTCD		-2.34	
GSTP1	-2.35	-2.06	
HIF1A	0.00	0.56	0.66
HMOX1	5.86	4.01	2.77
HOXA7	0.00	0.53	0.61
HSF1	0.00	0.00	2.51
HSP90AA1	3.29	2.26	2.13
HSP90AB1	2.76	2.30	2.18
HSPA13	2.03	2.53	
HSPA1B	4.87	2.69	
HSPA4	2.06	2.54	2.45
HSPA4L	2.14		
HSPA8	4.10	4.73	3.75
HSPA8	-5.41	-2.38	3.75
HSPA9	2.01	3.26	2.11
HSPB8	2.72	2.21	
HSPD1	2.80	2.56	2.43
IFNA2		-3.63	-3.97
IL10	5.19	5.33	5.69
IL10RB	4.31	4.47	
IL11	8.31	4.64	3.99
IL12B	3.32	2.64	2.34
IL12RB	3.96	2.24	
IL15RA	2.08		
IL16	2.82		
IL1B	7.54	5.95	4.60
IL1R2	7.12	4.78	3.33
IL1RAPL1	2.59	5.07	2.39
IL1RAPL2	8.31	6.10	3.83

HGNC symbol	Severe	Moderate	Mild
IL20RA	2.41		
IL21R	3.36	3.09	2.70
IL2RG	2.47		
IL4	-2.33	-2.06	
IL6R	2.07		
IL8	5.57	3.44	2.46
ILVBL	-2.12	-2.02	
IP6K2	2.68		
ITGA6	0.39	0.54	0.42
ITGB1	0.00	0.53	0.52
LALBA	-3.13		
LEP	0.00	2.16	0.00
LGALS1	2.13		
LTB	8.51	3.97	3.35
LTB4R	3.73	2.69	2.24
MBL2	4.00		
MGST1	-2.92	-2.48	
MHCI	-4.39		
MLKL	2.32	2.51	
MMP13	4.30	6.43	5.57
MMP19	5.90	5.63	1.28
MMP3	3.46	4.69	4.60
MMP9	3.06	3.04	3.55
MUC16	3.19		
MUC2	-4.63		
MUC21	3.11		
MUC5B	-90.20		
MUC7	-3.01		
MYH9	0.00	0.79	0.00
NFE2L1	2.26	2.02	-2.87
NFKBIA	2.23		
NOS2	3.39		
PARP12	-2.08		
PDK2			-2.02
PIK3CG	0.00	-1.26	0.00
PLSCR1		-2.06	
PTK2B	2.24		
RHOA	0.00	1.24	0.00
RIPK3	-3.09		
SAA2	3.82		
SHH	3.64	5.31	5.34
SLC12A4	2.60		
SLC14A2		-2.28	
SLC16A8	5.97		
SLC20A1	5.91	2.33	2.25
SLC25A17	-2.59	-2.34	
SLC25A22	4.59	3.67	2.97
SLC25A33	2.65		
SLC26A5	7.00	3.91	3.10
SLC2A1	2.47		
SLC2A13			-2.98
SLC2A4	2.67	2.27	
SLC30A4		-5.45	
SLC35A1	-2.22		
SLC38A2	4.99	3.16	3.03
SLC39A1	2.53	3.34	2.70
SLC39A7	-4.18		
SLC3A2	3.04	2.28	
SLC51A	-3.24	-3.37	-3.98
SLC5A3	6.19	5.48	4.77
SLC6A6	2.17		
SLC7A2	-3.95	-3.91	
SLC9A3	-3.65	-3.05	-3.67
SLC9A3R1	2.57	4.03	2.72
SMAD3	2.56		
SMO	2.25		
SOCS3	5.02		

HGNC symbol	Severe	Moderate	Mild
ST6GALNAC1	-3.48		
ST6GALNAC2	5.25	10.14	
ST6GALNAC6	2.04		
TAB3	-46.98		
TGFBR2	0.00	-0.60	0.00
TIMP2	2.60	2.65	2.07
TIMP3	3.89	2.61	2.33
TLR3	-6.73		
TLR5	-3.11		
TMC5		-5.78	
TNFAIP6	-3.29		
TNFRSF6B	16.13	26.95	19.65
TNFRSF9	3.23		
TNFSF10	-2.37	-2.23	-2.33
TRAF1	2.44	2.20	2.07
TRAF3	2.95		
TRAF5	-2.16		
TRAF6	2.17	2.96	2.22
UGT1A6	2.16	2.14	
UQCR10	3.66		
UQCRFS1	-5.03		
ZNF205	-2.83		

B.5 Average fold change utilised in analysis of various SDEG transcripts is presented here. These were genes identified within the dataset as involved with biological processes of interest. Breakdown of the contribution of individual probes and description of the function of genes as additional information will enhance the level of information this table will convey.

Appendix C

Appendix C; Sampling metadata and additional information in community differences

C. 1 fieldwork raw data

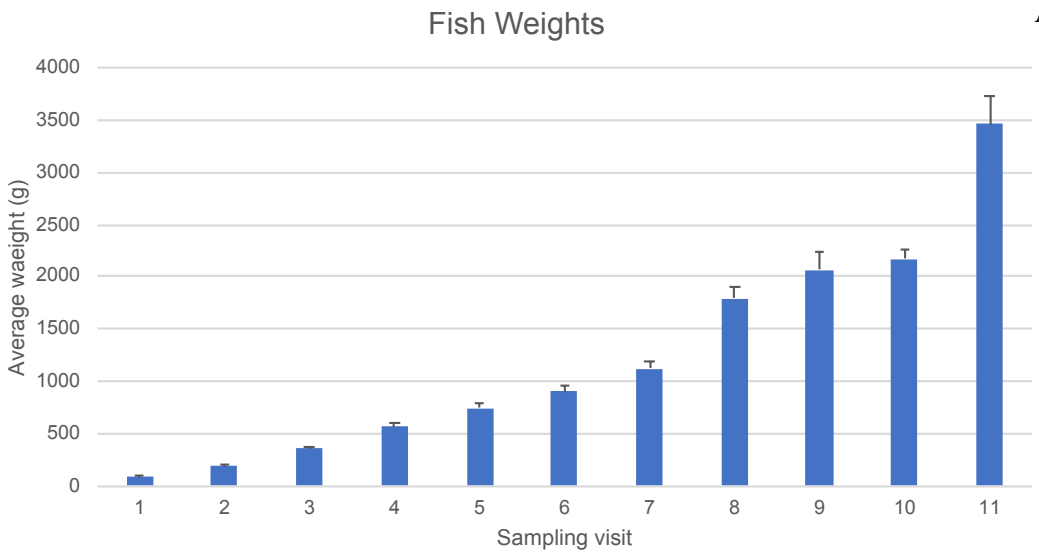
Sample	Epitheliostitis	AGD_positive	possible_autolysis	Bacteria	Histology_score	Histology classification	Group	Gross PGD	Gross AGD	Weight	Date	Cataracts	AGD class	PDG class
10F1	0	0	0	0	0	0 none	Ten	0	0	2300	23.03.18	none	none	none
10F10		0	0	0	0	2 none	Ten	0.5	0.5	1735	23.03.18	none	very_slight	very_slight
10F11		0	0	0	0	2 none	Ten	2	0	2040	23.03.18	none	none	localized
10F12		0	0	0	0	2 none	Ten	1	0	2495	23.03.18	none	none	very_slight
10F2		0	0	0	0	3 none	Ten	0	0.5	2095	23.03.18	none	very_slight	none
10F3		0	0	0	0	7 moderate	Ten	4	2	1830	23.03.18	none	localised	extensive
10F4		0	0	0	0	3 none	Ten	0	1	2215	23.03.18	none	very_slight	none
10F5		0	0	0	0	3 none	Ten	0.5	0.5	1965	23.03.18	none	very_slight	very_slight
10F6		0	0	0	0	3 none	Ten	1.5	0	2143	23.03.18	none	none	localized
10F7		0	0	0	0	3 none	Ten	1	0.5	2180	23.03.18	none	very_slight	very_slight
10F8		0	0	0	0	4 mild	Ten	1	0	2840	23.03.18	none	none	very_slight
10F9		0	0	0	0	4 mild	Ten	1	0	1860	23.03.18	none	none	very_slight
11F1		0	0	0	0	5 mild	Eleven	0.5	3	3450	23.05.18	none	multifocal	very_slight
11F11		0	0	0	0	5 mild	Eleven	2	3	4460	23.05.18	none	multifocal	localized
11F12		0	0	0	0	5 mild	Eleven	3	2	1990	23.05.18	none	localised	multifocal
11F2		0	0	0	0	2 none	Eleven	1	0	4523	23.05.18	none	none	very_slight
11F3		0	0	0	0	2 none	Eleven	1.5	0.5	4410	23.05.18	none	very_slight	localized
11F4		0	0	0	0	3 none	Eleven	1	1	3670	23.05.18	none	very_slight	very_slight
11F9		0	0	0	0	6 mild	Eleven	2	2	2343	23.05.18	none	localised	localized
1F1		0	0	0	0	0 none	One	1	1	87.9	17.05.17	one	none	very_slight
1F2		0	0	0	0	2 none	One	0	0	84.4	17.05.17	both	none	none
1F3		0	0	0	0	4 mild	One	2	0	106.8	17.05.17	none	none	localized
1F4		0	0	0	0	0 none	One	1	0	68	17.05.17	one	none	very_slight
1F5		0	0	0	0	1 none	One	0	0	74.8	17.05.17	none	none	none
1F6		0	0	0	0	0 none	One	1	0	120.6	17.05.17	none	none	very_slight
1F7		0	0	0	0	0 none	One	1	0	76.2	17.05.17	both	none	very_slight
1F8		0	0	0	0	3 none	One	1	0	74.3	17.05.17	one	none	very_slight
1F9		0	0	0	0	2 none	One	1	0	76.5	17.05.17	one	none	very_slight
2F1		1	1	0	0	1 none	Two	1	0	192	26.06.17	none	none	very_slight
2F10		0	0	1	0	3 none	Two	1.5	0	238	26.06.17	one	none	localized
2F11		0	0	0	0	4 mild	Two	1	0	189	26.06.17	one	none	very_slight
2F12		0	0	0	0	3 none	Two	1	0	166	26.06.17	both	none	very_slight
2F2		0	1	0	0	6 mild	Two	0	1	189	26.06.17	one	very_slight	none
2F3		0	0	0	0	1 none	Two	1.5	1	190	26.06.17	both	very_slight	localized
2F4		0	0	0	0	1 none	Two	0	0	230	26.06.17	both	none	none
2F5		0	0	0	0	2 none	Two	0	0	260	26.06.17	one	none	none
2F6		0	0	0	0	5 mild	Two	0	0	219	26.06.17	one	none	very_slight
2F7		0	0	0	0	2 none	Two	1	1	104	26.06.17	one	none	very_slight
2F8		0	0	1	0	1 none	Two	0	0	202	26.06.17	one	none	none
2F9		0	0	1	0	3 none	Two	1	0	133	26.06.17	both	none	very_slight
3F1		0	0	0	0	1 none	Three	0	0	331	26.07.18	none	none	none
3F10		0	0	0	0	4 mild	Three	1	1	420	26.07.18	both	very_slight	very_slight
3F11		0	0	0	0	2 none	Three	2	1.5	333	26.07.18	both	localised	localized
3F12		0	0	0	0	1 none	Three	2	0	328	26.07.18	both	none	localized

3F2	0	0	0	0	0	none	Three	1	0	331	26.07.18	both	none	very_slight
3F3	0	0	0	0	0	none	Three	0	1	381	26.07.18	both	very_slight	none
3F4	0	0	0	0	1	none	Three	1	0	510	26.07.18	both	none	very_slight
3F5	0	0	0	0	1	none	Three	2	1	371	26.07.18	none	very_slight	localised
3F6	0	0	0	0	0	none	Three	0	0	243	26.07.18	one	none	none
3F7	0	0	0	0			Three	0	1.5	310	26.07.18	both	localised	none
3F8	0	0	0	0	3	none	Three	1	0	282	26.07.18	one	none	very_slight
3F9	0	0	0	0	0	none	Three	1	0	310	26.07.18	both	none	very_slight
4F1	0	0	0	0	8	moderate	Four	3	0	536	07.09.17	none	none	multifocal
4F10	0	1	0	0	4	mild	Four	2	1	557	07.09.17	one	very_slight	localised
4F11	0	1	0	0	9	moderate	Four	2	2.5	302	07.09.17	none	multifocal	localised
4F12	0	0	0	0	4	mild	Four	1.5	3	762	07.09.17	none	multifocal	localised
4F2	0	0	0	0	9	moderate	Four	3	2	636	07.09.17	none	localised	multifocal
4F3	0	0	0	0	11	severe	Four	4	1	561	07.09.17	one	very_slight	extensive
4F4	0	0	0	0	6	mild	Four	1.5	2.5	487	07.09.17	none	multifocal	localised
4F5	0	1	0	0	10	severe	Four	3	0	384	07.09.17	none	none	multifocal
4F6	0	1	0	0	9	moderate	Four	2	0	712	07.09.17	none	none	localised
4F7	0	0	0	1	9	moderate	Four	4	1	531	07.09.17	one	very_slight	extensive
4F8	0	1	0	0	8	moderate	Four	2.5	1.5	540	07.09.17	one	localised	multifocal
4F9	0	0	0	0	9	moderate	Four	2	1	678	07.09.17	none	very_slight	localised
5F1	0	1	0	0	10	severe	Five	1	3	532	24.09.17	none	multifocal	very_slight
5F10	0	0	0	0	4	mild	Five	1	0	503	24.09.17	none	none	very_slight
5F11	0	1	0	0	11	severe	Five	2.5	1	796	24.09.17	none	very_slight	multifocal
5F12	0	0	0	0	6	mild	Five	3	1	850	24.09.17	none	very_slight	multifocal
5F2	0	0	0	0	7	moderate	Five	3	0	769	24.09.17	none	none	multifocal
5F3	0	0	0	0	6	mild	Five	1.5	0	695	24.09.17	none	none	localised
5F4	0	0	0	0	4	mild	Five	3	1	995	24.09.17	one	very_slight	multifocal
5F5	0	1	0	0	9	moderate	Five	0	1.5	612	24.09.17	none	localised	none
5F6	0	0	0	0	0	none	Five	2	0	855	24.09.17	none	none	localised
5F7	0	0	0	0	5	mild	Five	2.5	1	806	24.09.17	none	very_slight	multifocal
5F8	0	0	0	0	5	mild	Five	2	1	807	24.09.17	none	very_slight	localised
5F9	0	0	0	0	8	moderate	Five	4	2	628	24.09.17	none	localised	extensive
6F1	0	0	0	0	3	none	Six	2	0	1455	24.11.17	none	none	localised
6F10	0	0	0	0	3	none	Six	2	0	1003	24.11.17	none	none	localised
6F11	0	0	0	0	3	none	Six	3	1	760	24.11.17	none	very_slight	multifocal
6F12	0	0	0	0	2	none	Six	2	1	791	24.11.17	none	very_slight	localised
6F2	0	0	0	0	8	moderate	Six	1	0	875	24.11.17	one	none	very_slight
6F3	0	0	0	0	2	none	Six	1.5	0	801	24.11.17	none	none	localised
6F4	0	0	0	0	4	mild	Six	2	0	990	24.11.17	none	none	localised
6F5	0	0	0	0	5	mild	Six	3	0	853	24.11.17	none	none	multifocal
6F6	0	0	0	0	7	moderate	Six	2.5	1.5	750	24.11.17	none	localised	multifocal
6F7	0	0	0	0	4	mild	Six	4	1	1005	24.11.17	none	very_slight	extensive
6F8	0	0	0	0	5	mild	Six	3	2	752	24.11.17	one	localised	multifocal
6F9	0	0	0	0	4	mild	Six	3	2.5	760	24.11.17	none	multifocal	multifocal
7F1	0	0	0	0	7	moderate	Seven	1	1	1450	30.11.17	none	very_slight	very_slight
7F10	0	0	0	0	3	none	Seven	2.5	1	1160	30.11.17	none	very_slight	multifocal

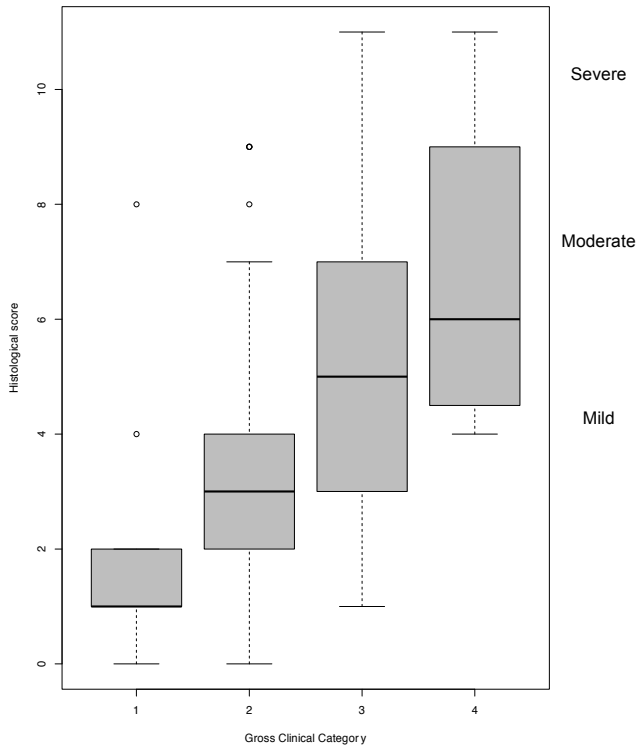
7F11	0	0	0	0	5	mild	Seven	1	1	790	30.11.17	none	very_slight	very_slight
7F12	1	0	0	0	8	moderate	Seven	0	0	1000	30.11.17	none	none	none
7F2	0	0	0	0	6	mild	Seven	0	1.5	805	30.11.17	none	localised	none
7F3	0	0	0	0	3	none	Seven	1	0	970	30.11.17	none	none	very_slight
7F4	1	0	0	0	2	none	Seven	0	0	1285	30.11.17	none	none	none
7F5	1	0	0	0	7	moderate	Seven	3	1	840	30.11.17	none	very_slight	multifocal
7F6	1	0	0	0	9	moderate	Seven	1	0	1720	30.11.17	none	none	very_slight
7F7	0	0	0	0	3	none	Seven	0	1	1250	30.11.17	none	very_slight	none
7F8	0	0	0	0	3	none	Seven	1	0	1030	30.11.17	none	none	very_slight
7F9	0	0	0	0	4	mild	Seven	1	0	1100	30.11.17	none	none	very_slight
8F1	0	0	0	0	1	none	Eight	0	0	2535	31.01.18	one	none	none
8F10	0	0	0	0	2	none	Eight	1.5	1	1985	31.01.18	none	very_slight	localised
8F11	0	0	0	0	5	mild	Eight	0.5	1	1908	31.01.18	none	very_slight	very_slight
8F12	0	0	0	0	4	mild	Eight	1	0	1625	31.01.18	none	none	very_slight
8F2	0	0	0	0	0	none	Eight	0.5	0	2584	31.01.18	none	none	very_slight
8F3	0	0	0	0	4	mild	Eight	1	1	1850	31.01.18	none	very_slight	very_slight
8F4	0	0	0	0	3	none	Eight	1	1	1400	31.01.18	one	very_slight	very_slight
8F5	0	0	0	0	0	none	Eight	0.5	0	1865	31.01.18	none	none	very_slight
8F6	0	0	0	0	3	none	Eight	1	0	2500	31.01.18	none	none	very_slight
8F7	1	0	0	0	6	mild	Eight	1.5	1	1350	31.01.18	none	very_slight	localised
8F8	0	0	0	0	6	mild	Eight	1	0	1820	31.01.18	none	none	very_slight
8F9	0	0	0	0	6	mild	Eight	1.5	0	1640	31.01.18	none	none	localised
9F1	0	0	0	0	4	mild	Nine	0.5	0	2365	8.03.18	none	none	very_slight
9F10	0	0	0	0	3	none	Nine	1	0	2230	8.03.18	none	none	very_slight
9F11	0	0	0	0	3	none	Nine	1	1.5	3240	8.03.18	none	localised	very_slight
9F12	0	0	0	0	5	mild	Nine	1	2	1630	8.03.18	none	localised	very_slight
9F2	0	0	0	0	0	none	Nine	0.5	0	1310	8.03.18	one	none	very_slight
9F3	0	0	0	0	2	none	Nine	1	0	1700	8.03.18	none	none	very_slight
9F4	0	0	0	0	2	none	Nine	1	0	1835	8.03.18	none	none	very_slight
9F5	0	0	0	0	4	mild	Nine	1.5	0.5	2360	8.03.18	none	very_slight	localised
9F6	0	0	0	0	5	mild	Nine	1	0	1430	8.03.18	none	none	very_slight
9F7	0	0	0	0	3	none	Nine	1.5	0	2910	8.03.18	none	none	localised
9F8	0	0	0	0	4	mild	Nine	0	0	1640	8.03.18	none	none	none
9F9	0	0	0	0	4	mild	Nine	0.5	2	2160	8.03.18	none	localised	very_slight

C.2 Fish weight and histology

A



B

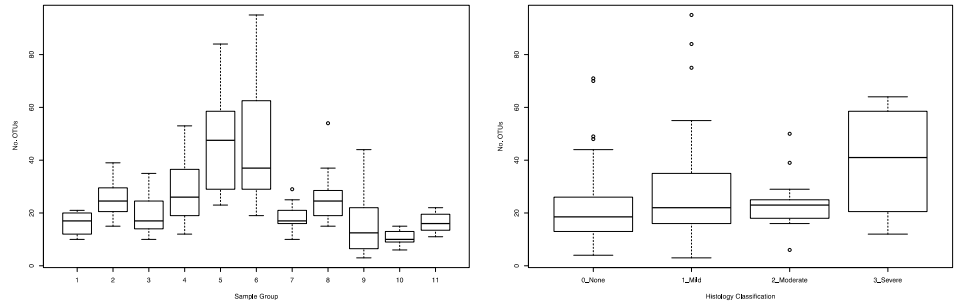


C.2: A: Mean fish weight throughout study period. B: Association of obtained histology and gross pathology scores for all samples. Gross clinical category is a composite of gross AGD and general gill scores. Results appear mainly in agreement, with high histology scoring fish also obtaining high gross scores.

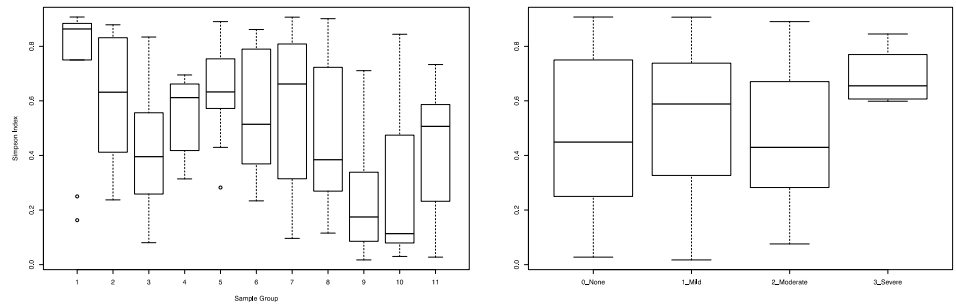
C.3 Diversity indices

Richness

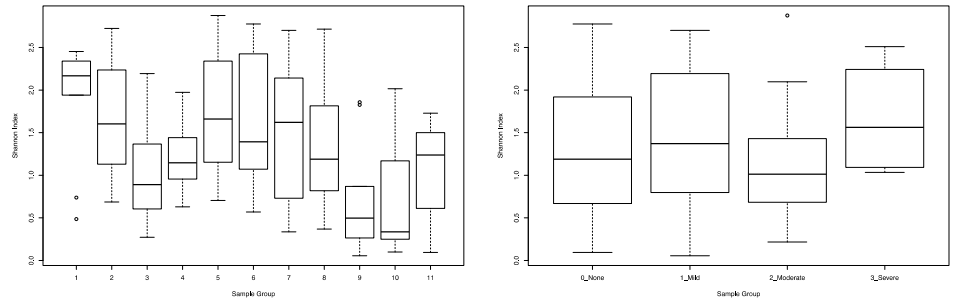
A B



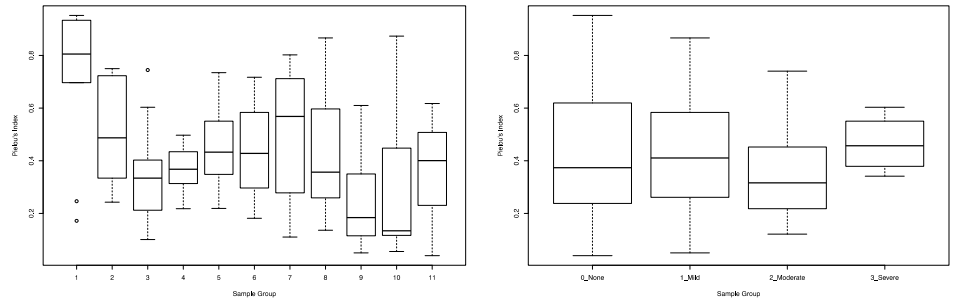
Simpson Index (species diversity)



Shannon Index (species diversity)



Pielou's Index (species evenness)



C.3 Diversity indices for gill biopsies calculated for fish by group (A) and histological classification (B). Seasonal trends are apparent with varied sampling data, however no clear pattern is apparently with histological score.

C.4 ANOSIM results by group

R values

	One	Two	Three	Four	Five	Six	Seven	Eight	Nine	Ten	Eleven
One											
Two	0.466										
Three	0.826	0.842									
Four	0.805	0.961	0.396								
Five	0.707	0.956	0.816	0.169							
Six	0.776	0.913	0.226	0.385	0.555						
Seven	0.679	0.899	0.131	0.133	0.341	0.07					
Eight	0.816	0.945	0.046	0.341	0.659	0.12	0.04				
Nine	0.904	0.978	0.046	0.484	0.842	0.284	0.192	0.077			
Ten	0.797	0.903	0.065	0.381	0.636	0.214	0.135	0.09	-0.005		
Eleven	0.481	0.853	0.62	0.665	0.72	0.422	0.456	0.518	0.581	0.464	

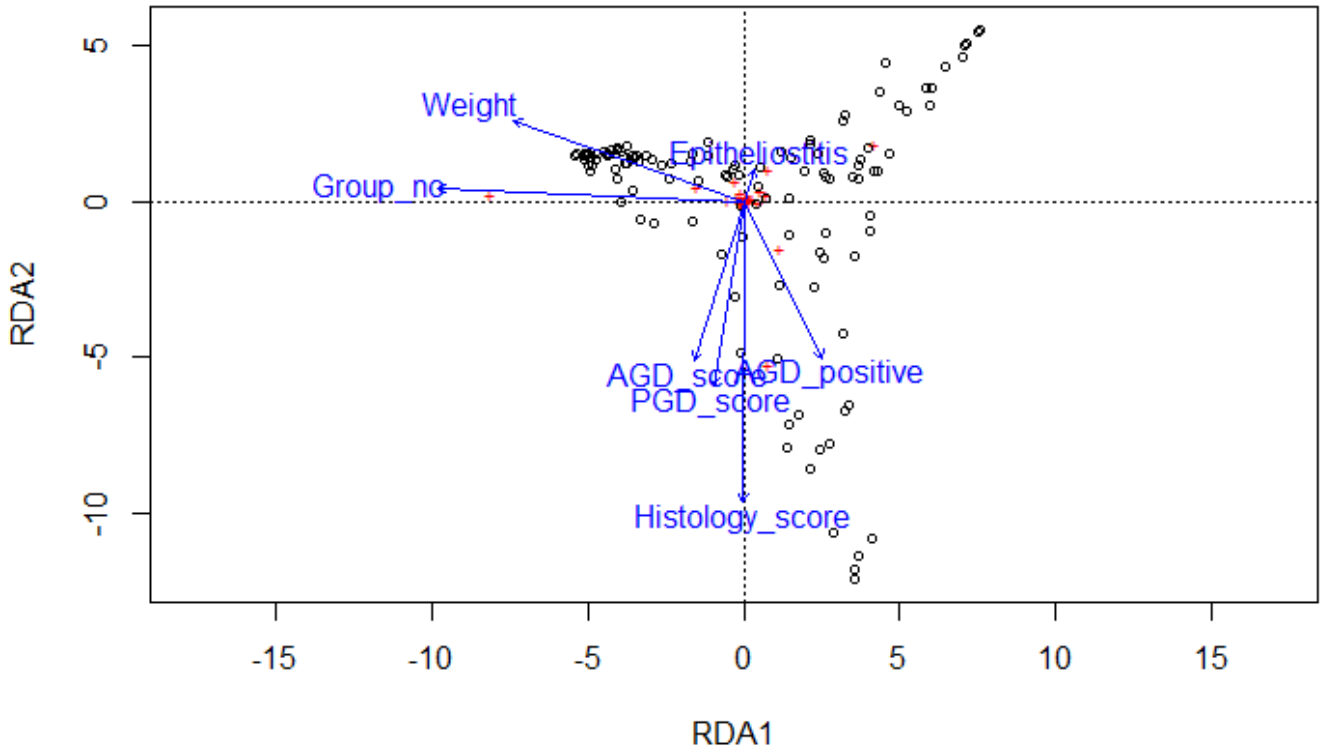
p value

	One	Two	Three	Four	Five	Six	Seven	Eight	Nine	Ten	Eleven
One											
Two	**0.001**										
Three	**0.001**	**0.001**									
Four	**0.001**	**0.001**	**0.001**								
Five	**0.002**	**0.001**	**0.001**	0.011							
Six	**0.001**	**0.001**	**0.002**	**0.002**	**0.001**						
Seven	**0.001**	**0.001**	0.02	0.028	**0.001**	0.08					
Eight	**0.001**	**0.001**	0.087	**0.002**	**0.001**	0.015	0.146				
Nine	**0.001**	**0.001**	0.079	**0.001**	**0.001**	**0.001**	**0.007**	0.039			
Ten	**0.001**	**0.001**	0.044	**0.001**	**0.001**	**0.002**	0.025	0.026	0.435		
Eleven	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	**0.007**	**0.004**	**0.003**	**0.002**	**0.003**	

C.4: Uses of Primer 7 ANOSIM function. R values representing strength of factors on the significance, and p values indicating the significance levels of the variation.

C.5 Redundancy analysis

A



B

Variables	RDA1	RDA2	RDA3	RDA4	RDA5	RDA6
Epitheliocystis (in histology)	0.032893	0.10034	-0.11028	0.08096	-0.21079	0.38911
AGD positive (in histology)	0.24201	0.48015	0.04571	0.14629	0.07292	**-0.69649**
Histology score	-0.001618	**-0.92035**	0.21225	-0.10530	-0.21691	0.12367
PGD score (gross)	-0.092599	-0.56557	0.15694	**0.71509**	0.05544	0.31686
AGD score (gross)	-0.153911	-0.48930	0.34864	-0.12443	**0.74479**	0.16756
Weight (g)	-0.709991	0.24780	**0.62539**	-0.13510	-0.12473	-0.07546
Group number / Sampling data	**-0.938844**	0.04044	0.26499	-0.16186	-0.09879	-0.02680

C.5 Redundancy (RDA) analysis of microbial dataset ordinated by components of greatest explainable variation. Correlation triplot (A) and plot scores for constraining variables (B) illustrated. In part A, explanatory variables are represented by blue lines, bacterial species (response variables) by red crosses, and samples by black points. In part B, plot scores for constraining variables are presented. Output of RDA analysis suggests total constrained inertia observed accounted for relatively little variance observed (23%), breaking down to 15.3%, 5.94% and 1.6% for the measured variables with greatest proportionate contribution to variation (RDA1-3). Explanatory variables associated with fish size and timing of sampling appear to account for the greatest variation along the x-axis (RDA1), with explanatory variables associated with gill structure and pathology accounting for variation along the y axis (RDA2). The angle of lines in RDA analysis is a graphical representation of the correlation of explanatory variables, and so this figure demonstrates a correlation of gross and histological scores

with associated microbial community structure differences. Fish weight and sample group (both variables linked to timing of sampling) also show correlation. This RDA was performed through use of vegan package in R (rda and plot functions).

C.6 SIMPER analysis

Groups none & mild
Average dissimilarity = 68.76

Species	Group none Av.Abund	Group mild Av.Abund	Av.Diss	Diss/SD	Contrib%
D_0__Bacteria;D_1__Proteobacteria;D_2__Betaproteobacteria;Procabacteriales;Procabacteriaceae;__	44.37	47.49	21.13	1.37	30.73
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Alteromonadales;D_4__Shewanellaceae;D_5__Shewanella	12.39	2.62	6.87	0.56	10
D_0__Bacteria;D_1__Proteobacteria;D_2__Betaproteobacteria;D_3__Nitrosomonadales;D_4__Nitrosomonadaceae;D_5__Candidatus Branchiomonas	2.01	12.21	6.34	0.61	9.22
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Pseudomonadales;D_4__Moraxellaceae;D_5__Psychrobacter	4.46	6.26	3.85	0.85	5.6
D_0__Bacteria;D_1__Verrucomicrobia;D_2__Verrucomicrobiae;D_3__Verrucomicrobiales;D_4__Rubritaleaceae;D_5__Rubritalea	3.35	5.64	3.77	0.57	5.48
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Alteromonadales;D_4__Pseudoalteromonadaceae;D_5__Pseudoalteromonas	3.92	4.18	3.56	0.43	5.18
D_0__Bacteria;D_1__Chlamydiae;D_2__Chlamydiae;D_3__Chlamydiales;D_4__Chlamydiales Incertae Sedis;D_5__Candidatus Piscichlamydia	5.38	1.7	3.1	0.45	4.51
D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Rhodobacterales;D_4__Rhodobacteraceae;__	2.06	1.24	1.45	0.52	2.1
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Enterobacteriales;D_4__Enterobacteriaceae;D_5__Serratia	2.13	1.57	1.25	0.76	1.82
D_0__Bacteria;D_1__Bacteroidetes;D_2__Flavobacteriia;D_3__Flavobacteriales;D_4__Flavobacteriaceae;D_5__Tenacibaculum	1.82	0.8	1.24	0.2	1.8
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Pseudomonadales;D_4__Pseudomonadaceae;D_5__Pseudomonas	1.73	0.82	1.08	0.4	1.57
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Enterobacteriales;D_4__Enterobacteriaceae;D_5__Escherichia-Shigella	0.02	1.96	0.99	0.16	1.44
D_0__Bacteria;D_1__Bacteroidetes;D_2__Flavobacteriia;D_3__Flavobacteriales;D_4__Flavobacteriaceae;D_5__Flavobacterium	1.12	0.75	0.81	0.51	1.18
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Oceanospirillales;D_4__Hahellaceae;D_5__Endozoicomonas	1.2	0	0.6	0.13	0.88
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Vibrionales;D_4__Vibrionaceae;D_5__Vibrio	0.47	0.67	0.53	0.32	0.77
D_0__Bacteria;D_1__Bacteroidetes;D_2__Flavobacteriia;D_3__Flavobacteriales;D_4__Flavobacteriaceae;__	0.64	0.49	0.5	0.36	0.73
D_0__Bacteria;D_1__Chlamydiae;D_2__Chlamydiae;D_3__Chlamydiales;D_4__Chlamydiaceae;D_5__Candidatus Clavichlamydia	0.32	0.57	0.44	0.22	0.64
D_0__Bacteria;D_1__Bacteroidetes;D_2__Flavobacteriia;D_3__Flavobacteriales;D_4__Flavobacteriaceae;D_5__Chryseobacterium	0.39	0.59	0.43	0.54	0.62
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Pseudomonadales;D_4__Moraxellaceae;D_5__Acinetobacter	0.68	0.25	0.38	0.46	0.56
D_0__Bacteria;D_1__Firmicutes;D_2__Bacilli;D_3__Lactobacillales;D_4__Enterococcaceae;D_5__Enterococcus	0.08	0.69	0.38	0.18	0.55
D_0__Bacteria;D_1__Proteobacteria;D_2__Betaproteobacteria;D_3__Burkholderiales;D_4__Oxalobacteraceae;D_5__Herbaspirillum	0.55	0.09	0.31	0.28	0.44
D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Rhodobacterales;D_4__Rhodobacteraceae;D_5__Sulfitobacter	0.04	0.55	0.29	0.25	0.42
D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Sphingomonadales;D_4__Sphingomonadaceae;__	0.26	0.34	0.25	0.63	0.36
D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Sphingomonadales;D_4__Sphingomonadaceae;D_5__Sphingomonas	0.32	0.22	0.23	0.42	0.33
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Gammaproteobacteria Incertae Sedis;D_4__Unknown Family;D_5__uncultured	0.33	0.13	0.22	0.24	0.32
D_0__Bacteria;D_1__Proteobacteria;D_2__Betaproteobacteria;D_3__Burkholderiales;D_4__Comamonadaceae;D_5__Aquabacterium	0.35	0.08	0.21	0.24	0.31
D_0__Bacteria;D_1__Firmicutes;D_2__Bacilli;D_3__Lactobacillales;D_4__Lactobacillaceae;D_5__Lactobacillus	0.18	0.25	0.21	0.24	0.3
D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Rhizobiales;D_4__Methylobacteriaceae;D_5__Methylobacterium	0.34	0.08	0.2	0.18	0.29
D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Rhizobiales;D_4__Phyllobacteriaceae;D_5__Mesorhizobium	0.36	0.04	0.2	0.22	0.29
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Pseudomonadales;D_4__Moraxellaceae;D_5__Perlucidibaca	0.31	0.09	0.19	0.26	0.28
D_0__Bacteria;D_1__Proteobacteria;D_2__Betaproteobacteria;D_3__Burkholderiales;D_4__Oxalobacteraceae;D_5__Janthinobacterium	0.28	0.12	0.19	0.3	0.28
D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Rhodobacterales;D_4__Rhodobacteraceae;D_5__Paracoccus	0.14	0.26	0.19	0.37	0.27
D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Rhodobacterales;D_4__Rhodobacteraceae;D_5__Loktanella	0.17	0.2	0.17	0.4	0.25
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Vibrionales;D_4__Vibrionaceae;D_5__Photobacterium	0.13	0.23	0.17	0.34	0.24
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Xanthomonadales;D_4__JTB255 marine benthic group;__	0.13	0.22	0.17	0.35	0.24

Groups none & moderate
Average dissimilarity = 68.58

Species	Group none Av.Abund	Group moder Av.Abund	Av.Diss	Diss/SD	Contrib%
D_0__Bacteria;D_1__Proteobacteria;D_2__Betaproteobacteria;Procabacteriales;Procabacteriaceae;__	44.37	46.31	19.99	1.44	29.14
D_0__Bacteria;D_1__Proteobacteria;D_2__Betaproteobacteria;D_3__Nitrosomonadales;D_4__Nitrosomonadaceae;D_5__Candidatus Branchiomonas	2.01	28.86	13.93	0.93	20.31
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Alteromonadales;D_4__Shewanellaceae;D_5__Shewanella	12.39	2.37	6.74	0.56	9.83
D_0__Bacteria;D_1__Verrucomicrobia;D_2__Verrucomicrobiae;D_3__Verrucomicrobiales;D_4__Rubritaleaceae;D_5__Rubritalea	3.35	8.54	4.65	0.91	6.78
D_0__Bacteria;D_1__Chlamydiae;D_2__Chlamydiae;D_3__Chlamydiales;D_4__Chlamydiales Incertae Sedis;D_5__Candidatus Piscichlamydia	5.38	4.04	4.05	0.53	5.91
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Pseudomonadales;D_4__Moraxellaceae;D_5__Psychrobacter	4.46	1.7	2.38	0.69	3.47
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Alteromonadales;D_4__Pseudoalteromonadaceae;D_5__Pseudoalteromonas	3.92	0.54	2.09	0.32	3.04
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Enterobacteriales;D_4__Enterobacteriaceae;D_5__Serratia	2.13	1.07	1.11	0.71	1.62
D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Rhodobacterales;D_4__Rhodobacteraceae;__	2.06	0.33	1.09	0.48	1.59
D_0__Bacteria;D_1__Bacteroidetes;D_2__Flavobacteriia;D_3__Flavobacteriales;D_4__Flavobacteriaceae;D_5__Tenacibaculum	1.82	0.28	1.02	0.17	1.49
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Pseudomonadales;D_4__Pseudomonadaceae;D_5__Pseudomonas	1.73	0.21	0.9	0.34	1.31
D_0__Bacteria;D_1__Bacteroidetes;D_2__Flavobacteriia;D_3__Flavobacteriales;D_4__Flavobacteriaceae;D_5__Flavobacterium	1.12	0.35	0.67	0.44	0.98
D_0__Bacteria;D_1__Bacteroidetes;D_2__Flavobacteriia;D_3__Flavobacteriales;D_4__Flavobacteriaceae;__	0.64	0.78	0.64	0.4	0.94
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Oceanospirillales;D_4__Hahellaceae;D_5__Endozoicomonas	1.2	0	0.6	0.13	0.88
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Pseudomonadales;D_4__Moraxellaceae;D_5__Acinetobacter	0.68	0.42	0.42	0.52	0.62
D_0__Bacteria;D_1__Proteobacteria;D_2__Betaproteobacteria;D_3__Burkholderiales;D_4__Oxalobacteraceae;D_5__Herbaspirillum	0.55	0.06	0.29	0.26	0.43
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Vibrionales;D_4__Vibrionaceae;D_5__Vibrio	0.47	0.14	0.29	0.24	0.42
D_0__Bacteria;D_1__Bacteroidetes;D_2__Flavobacteriia;D_3__Flavobacteriales;D_4__Flavobacteriaceae;D_5__Chryseobacterium	0.39	0.13	0.24	0.32	0.34
D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Rhizobiales;D_4__Phyllobacteriaceae;D_5__Mesorhizobium	0.36	0.01	0.18	0.2	0.27
D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Sphingomonadales;D_4__Sphingomonadaceae;D_5__Sphingomonas	0.32	0.06	0.18	0.33	0.26
D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Rhizobiales;D_4__Methylobacteriaceae;D_5__Methylobacterium	0.34	0.03	0.18	0.16	0.26
D_0__Bacteria;D_1__Proteobacteria;D_2__Betaproteobacteria;D_3__Burkholderiales;D_4__Comamonadaceae;D_5__Aquabacterium	0.35	0	0.18	0.2	0.26

Groups none & severe
Average dissimilarity = 82.86

Species	Group none Av.Abund	Group severe Av.Abund	Av.Diss	Diss/SD	Contrib%
D_0__Bacteria;D_1__Proteobacteria;D_2__Betaproteobacteria;D_3__Nitrosomonadales;D_4__Nitrosomonadaceae;D_5__Candidatus Branchiomonas	2.01	42.84	20.41	3.66	24.64
D_0__Bacteria;D_1__Proteobacteria;D_2__Betaproteobacteria;Procabacteriales;Procabacteriaceae;__	44.37	14.1	19.8	1.37	23.89
D_0__Bacteria;D_1__Verrucomicrobia;D_2__Verrucomicrobiae;D_3__Verrucomicrobiales;D_4__Rubritaleaceae;D_5__Rubritalea	3.35	24.89	11.83	1.44	14.27
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Alteromonadales;D_4__Shewanellaceae;D_5__Shewanella	12.39	2.78	6.79	0.57	8.2
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Pseudomonadales;D_4__Moraxellaceae;D_5__Psychrobacter	4.46	4.56	3.22	0.94	3.88
D_0__Bacteria;D_1__Chlamydiae;D_2__Chlamydiae;D_3__Chlamydiales;D_4__Chlamydiales Incertae Sedis;D_5__Candidatus Piscichlamydia	5.38	0.06	2.69	0.38	3.25
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Alteromonadales;D_4__Pseudoalteromonadaceae;D_5__Pseudoalteromonas	3.92	0.81	2.11	0.32	2.54
D_0__Bacteria;D_1__Chlamydiae;D_2__Chlamydiae;D_3__Chlamydiales;D_4__Simkaniaceae;D_5__Candidatus Fritschea	0.06	2.11	1.06	0.79	1.28
D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Rhodobacterales;D_4__Rhodobacteraceae;__	2.06	0.1	1.03	0.45	1.25
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Enterobacteriales;D_4__Enterobacteriaceae;D_5__Serratia	2.13	0.27	0.98	0.61	1.19
D_0__Bacteria;D_1__Bacteroidetes;D_2__Flavobacteriia;D_3__Flavobacteriales;D_4__Flavobacteriaceae;D_5__Tenacibaculum	1.82	0.02	0.92	0.15	1.1
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Pseudomonadales;D_4__Pseudomonadaceae;D_5__Pseudomonas	1.73	0.35	0.9	0.34	1.08
D_0__Bacteria;D_1__Bacteroidetes;D_2__Flavobacteriia;D_3__Flavobacteriales;D_4__Flavobacteriaceae;D_5__Flavobacterium	1.12	0.33	0.63	0.44	0.76
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Oceanospirillales;D_4__Hahellaceae;D_5__Endozoicomonas	1.2	0	0.6	0.13	0.73
D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Rhodobacterales;D_4__Rhodobacteraceae;D_5__Loktanella	0.17	0.71	0.4	0.65	0.49
D_0__Bacteria;D_1__Bacteroidetes;D_2__Flavobacteriia;D_3__Flavobacteriales;D_4__Flavobacteriaceae;D_5__Chryseobacterium	0.39	0.47	0.38	0.51	0.46
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Pseudomonadales;D_4__Moraxellaceae;D_5__Acinetobacter	0.68	0.3	0.38	0.46	0.45
D_0__Bacteria;D_1__Bacteroidetes;D_2__Flavobacteriia;D_3__Flavobacteriales;D_4__Flavobacteriaceae;__	0.64	0.04	0.33	0.25	0.39
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Vibrionales;D_4__Vibrionaceae;D_5__Vibrio	0.47	0.17	0.3	0.25	0.36

Groups mild & moderate
Average dissimilarity = 61.30

Species	Group moder. Av.Abund	Group mild Av.Abund	Av.Diss	Diss/SD	Contrib%
D_0__Bacteria;D_1__Proteobacteria;D_2__Betaproteobacteria;Procabacteriales;Procabacteriaceae;__	46.31	47.49	19.07	1.42	31.12
D_0__Bacteria;D_1__Proteobacteria;D_2__Betaproteobacteria;D_3__Nitrosomonadales;D_4__Nitrosomonadaceae;D_5__Candidatus Branchiomonas	28.86	12.21	14.82	1.03	24.18
D_0__Bacteria;D_1__Verrucomicrobia;D_2__Verrucomicrobiae;D_3__Verrucomicrobiales;D_4__Rubritaleaceae;D_5__Rubritalea	8.54	5.64	4.96	0.95	8.09
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Pseudomonadales;D_4__Moraxellaceae;D_5__Psychrobacter	1.7	6.26	3.03	0.72	4.94
D_0__Bacteria;D_1__Chlamydiae;D_2__Chlamydiae;D_3__Chlamydiales;D_4__Chlamydiales Incertae Sedis;D_5__Candidatus Piscichlamydia	4.04	1.7	2.51	0.53	4.1
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Alteromonadales;D_4__Pseudoalteromonadaceae;D_5__Pseudoalteromonas	0.54	4.18	2.2	0.36	3.58
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Alteromonadales;D_4__Shewanellaceae;D_5__Shewanella	2.37	2.62	2.06	0.61	3.35
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Enterobacteriales;D_4__Enterobacteriaceae;D_5__Escherichia-Shigella	0.02	1.96	0.99	0.16	1.62
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Enterobacteriales;D_4__Enterobacteriaceae;D_5__Serratia	1.07	1.57	0.9	0.73	1.47
D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Rhodobacterales;D_4__Rhodobacteraceae;__	0.33	1.24	0.72	0.35	1.18
D_0__Bacteria;D_1__Bacteroidetes;D_2__Flavobacteriia;D_3__Flavobacteriales;D_4__Flavobacteriaceae;__	0.78	0.49	0.56	0.46	0.92
D_0__Bacteria;D_1__Bacteroidetes;D_2__Flavobacteriia;D_3__Flavobacteriales;D_4__Flavobacteriaceae;D_5__Tenacibaculum	0.28	0.8	0.49	0.5	0.8
D_0__Bacteria;D_1__Bacteroidetes;D_2__Flavobacteriia;D_3__Flavobacteriales;D_4__Flavobacteriaceae;D_5__Flavobacterium	0.35	0.75	0.49	0.49	0.8
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Pseudomonadales;D_4__Pseudomonadaceae;D_5__Pseudomonas	0.21	0.82	0.45	0.51	0.73
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Vibrionales;D_4__Vibrionaceae;D_5__Vibrio	0.14	0.67	0.38	0.31	0.61
D_0__Bacteria;D_1__Firmicutes;D_2__Bacilli;D_3__Lactobacillales;D_4__Enterococcaceae;D_5__Enterococcus	0	0.69	0.34	0.16	0.56
D_0__Bacteria;D_1__Bacteroidetes;D_2__Flavobacteriia;D_3__Flavobacteriales;D_4__Flavobacteriaceae;D_5__Chryseobacterium	0.13	0.59	0.31	0.66	0.5
D_0__Bacteria;D_1__Chlamydiae;D_2__Chlamydiae;D_3__Chlamydiales;D_4__Chlamydiaceae;D_5__Candidatus Clavichlamydia	0	0.57	0.29	0.16	0.47
D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Rhodobacterales;D_4__Rhodobacteraceae;D_5__Sulfitobacter	0.03	0.55	0.29	0.25	0.47
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Pseudomonadales;D_4__Moraxellaceae;D_5__Acinetobacter	0.42	0.25	0.23	0.99	0.38
D_0__Bacteria;D_1__Chlamydiae;D_2__Chlamydiae;D_3__Chlamydiales;D_4__Simkaniaceae;D_5__Candidatus Fritschea	0.25	0.17	0.19	0.48	0.3

Groups mild & severe
Average dissimilarity = 70.95

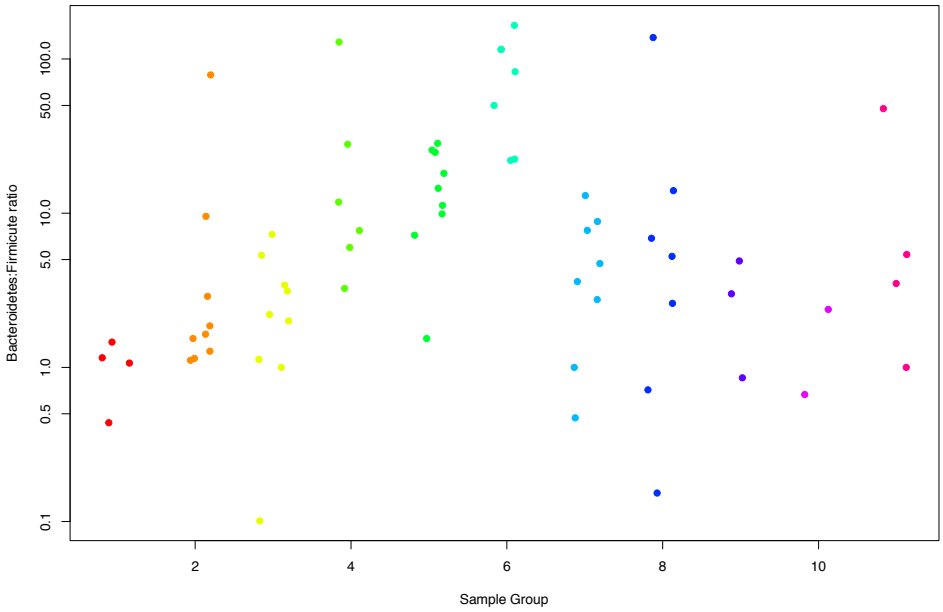
Species	Group mild Av.Abund	Group severe Av.Abund	Av.Diss	Diss/SD	Contrib%
D_0__Bacteria;D_1__Proteobacteria;D_2__Betaproteobacteria;Procabacteriales;Procabacteriaceae;__	47.49	14.1	19.32	1.28	27.22
D_0__Bacteria;D_1__Proteobacteria;D_2__Betaproteobacteria;D_3__Nitrosomonadales;D_4__Nitrosomonadaceae;D_5__Candidatus Branchiomonas	12.21	42.84	18.07	2.46	25.47
D_0__Bacteria;D_1__Verrucomicrobia;D_2__Verrucomicrobiae;D_3__Verrucomicrobiales;D_4__Rubritaleaceae;D_5__Rubritalea	5.64	24.89	11.24	1.37	15.85
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Pseudomonadales;D_4__Moraxellaceae;D_5__Psychrobacter	6.26	4.56	3.58	0.9	5.05
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Alteromonadales;D_4__Pseudoalteromonadaceae;D_5__Pseudoalteromonas	4.18	0.81	2.22	0.37	3.12
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Alteromonadales;D_4__Shewanellaceae;D_5__Shewanella	2.62	2.78	2.1	0.65	2.96
D_0__Bacteria;D_1__Chlamydiae;D_2__Chlamydiae;D_3__Chlamydiales;D_4__Simkaniaceae;D_5__Candidatus Fritschea	0.17	2.11	1.06	0.81	1.5
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Enterobacteriales;D_4__Enterobacteriaceae;D_5__Escherichia-Shigella	1.96	0	0.98	0.16	1.38
D_0__Bacteria;D_1__Chlamydiae;D_2__Chlamydiae;D_3__Chlamydiales;D_4__Chlamydiales Incertae Sedis;D_5__Candidatus Piscichlamydia	1.7	0.06	0.85	0.43	1.19
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Enterobacteriales;D_4__Enterobacteriaceae;D_5__Serratia	1.57	0.27	0.7	0.58	0.99
D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Rhodobacterales;D_4__Rhodobacteraceae;__	1.24	0.1	0.63	0.3	0.89
D_0__Bacteria;D_1__Bacteroidetes;D_2__Flavobacteriia;D_3__Flavobacteriales;D_4__Flavobacteriaceae;D_5__Flavobacterium	0.75	0.33	0.44	0.5	0.63
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Pseudomonadales;D_4__Pseudomonadaceae;D_5__Pseudomonas	0.82	0.35	0.44	0.53	0.62
D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Rhodobacterales;D_4__Rhodobacteraceae;D_5__Loktanella	0.2	0.71	0.41	0.66	0.58
D_0__Bacteria;D_1__Bacteroidetes;D_2__Flavobacteriia;D_3__Flavobacteriales;D_4__Flavobacteriaceae;D_5__Chryseobacterium	0.59	0.47	0.41	0.84	0.57
D_0__Bacteria;D_1__Bacteroidetes;D_2__Flavobacteriia;D_3__Flavobacteriales;D_4__Flavobacteriaceae;D_5__Tenacibaculum	0.8	0.02	0.4	0.42	0.56
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Vibrionales;D_4__Vibrionaceae;D_5__Vibrio	0.67	0.17	0.37	0.31	0.53
D_0__Bacteria;D_1__Firmicutes;D_2__Bacilli;D_3__Lactobacillales;D_4__Enterococcaceae;D_5__Enterococcus	0.69	0	0.34	0.16	0.49
D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Rhodobacterales;D_4__Rhodobacteraceae;D_5__Sulfitobacter	0.55	0.08	0.3	0.26	0.42

Groups moderate & severe
Average dissimilarity = 57.68

Species	Group moder. Av.Abund	Group severe Av.Abund	Av.Diss	Diss/SD	Contrib%
D_0__Bacteria;D_1__Proteobacteria;D_2__Betaproteobacteria;Procabacteriales;Procabacteriaceae;__	46.31	14.1	17.48	1.28	30.31
D_0__Bacteria;D_1__Proteobacteria;D_2__Betaproteobacteria;D_3__Nitrosomonadales;D_4__Nitrosomonadaceae;D_5__Candidatus Branchiomonas	28.86	42.84	15.97	2.09	27.68
D_0__Bacteria;D_1__Verrucomicrobia;D_2__Verrucomicrobiae;D_3__Verrucomicrobiales;D_4__Rubritaleaceae;D_5__Rubritalea	8.54	24.89	9.68	1.26	16.78
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Pseudomonadales;D_4__Moraxellaceae;D_5__Psychrobacter	1.7	4.56	2.32	0.98	4.02
D_0__Bacteria;D_1__Chlamydiae;D_2__Chlamydiae;D_3__Chlamydiales;D_4__Chlamydiales Incertae Sedis;D_5__Candidatus Piscichlamydia	4.04	0.06	2.02	0.41	3.5
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Alteromonadales;D_4__Shewanellaceae;D_5__Shewanella	2.37	2.78	1.75	0.99	3.04
D_0__Bacteria;D_1__Chlamydiae;D_2__Chlamydiae;D_3__Chlamydiales;D_4__Simkaniaceae;D_5__Candidatus Fritschea	0.25	2.11	1.07	0.83	1.86
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Alteromonadales;D_4__Pseudoalteromonadaceae;D_5__Pseudoalteromonas	0.54	0.81	0.55	0.73	0.96
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Enterobacteriales;D_4__Enterobacteriaceae;D_5__Serratia	1.07	0.27	0.47	0.56	0.81
D_0__Bacteria;D_1__Bacteroidetes;D_2__Flavobacteriia;D_3__Flavobacteriales;D_4__Flavobacteriaceae;__	0.78	0.04	0.39	0.35	0.68
D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Rhodobacterales;D_4__Rhodobacteraceae;D_5__Loktanella	0.01	0.71	0.36	0.58	0.62

C.6: Results of SIMPER analysis for comparison of histological classifications and observation of the genus-level taxa with greater percentage contribution to observed variation. Results are largely in agree with PCA loading values, although a greater resolution of the influence of worsening gill pathology can be observed via SIMPER

C..7 Bacteroidete:Firmicute relative ratios across sampling groups



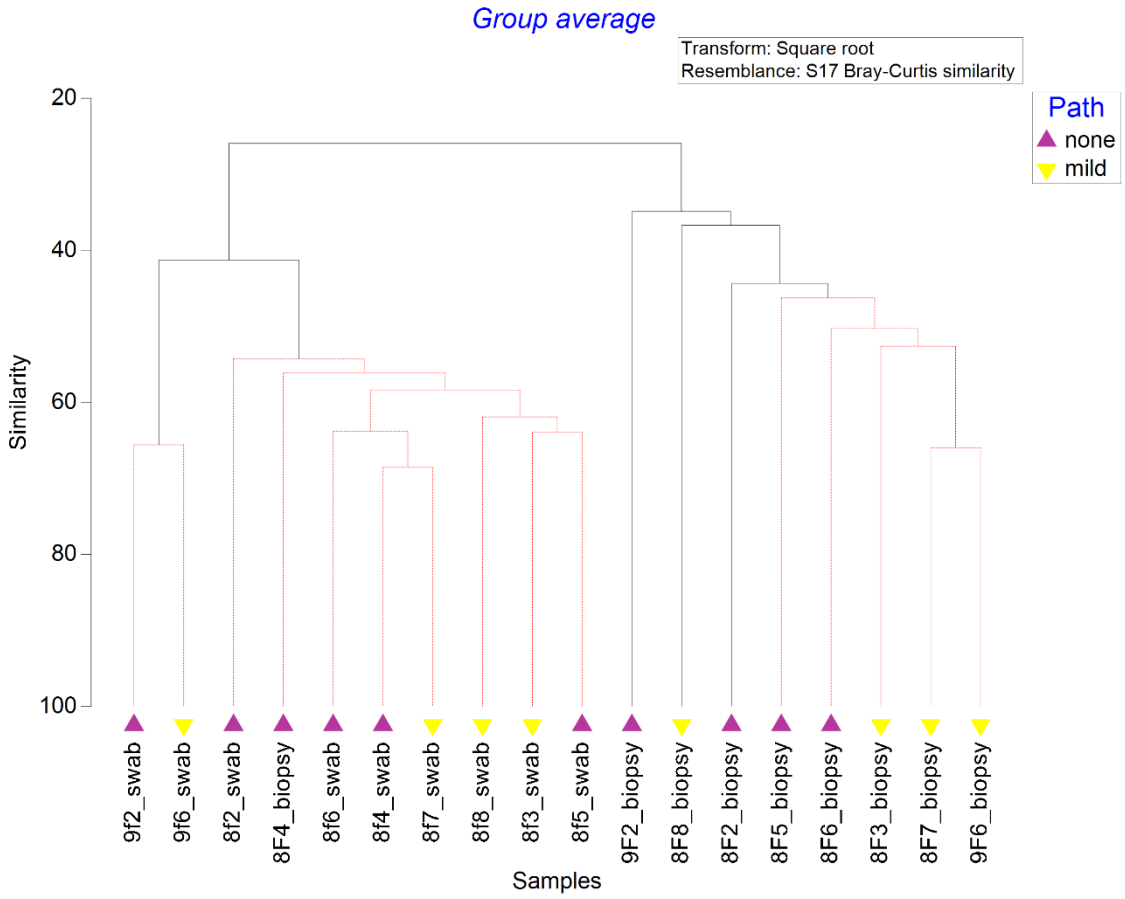
C.7: Variation in relative ratio between groups throughout sampling period. Benjamini-Hochberg corrected pairwise t-testing identifies a significant difference between groups 6 and 7 ($p = 0.00023$), between which H_2O_2 was applied to stock.

Identical figures were generated for every permutation of relative ratio between five dominant phyla for visual assessment alongside significance testing of observed variation. A clear change was apparent in this figure between group 6 and 7, proposed to be initiated by hydrogen peroxide treatment.

Appendix D

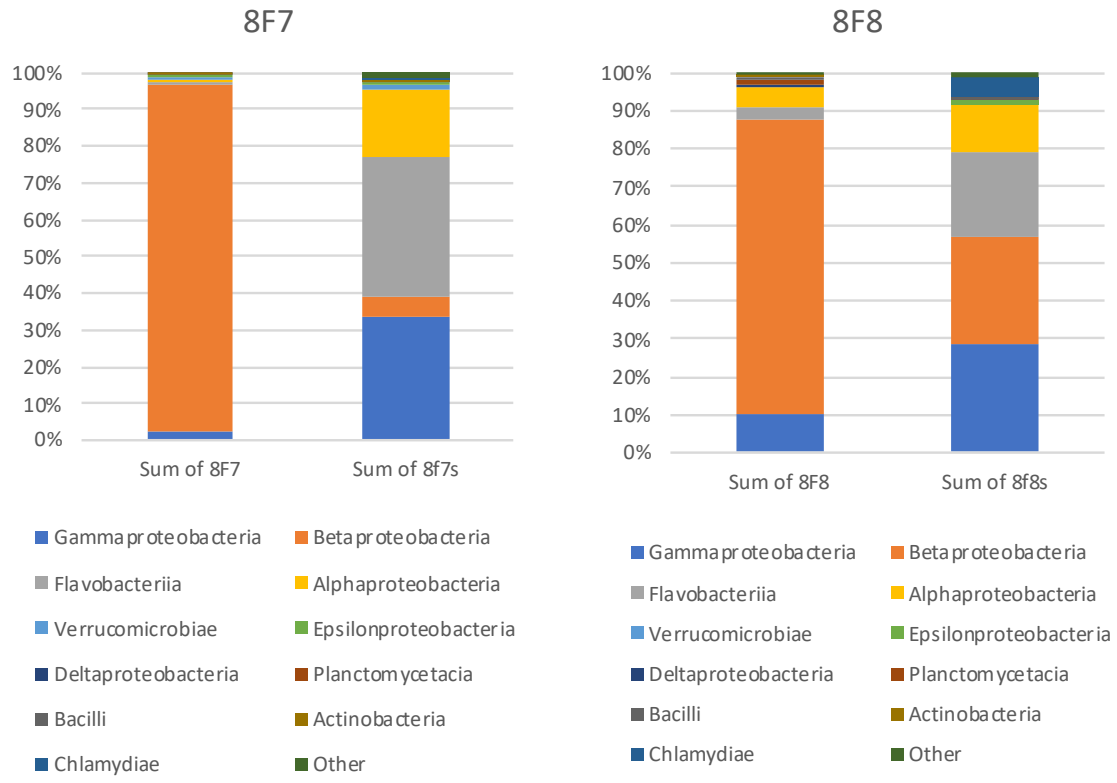
Appendix D: Additional visualization of differences in microbial community between swab and biopsy samples

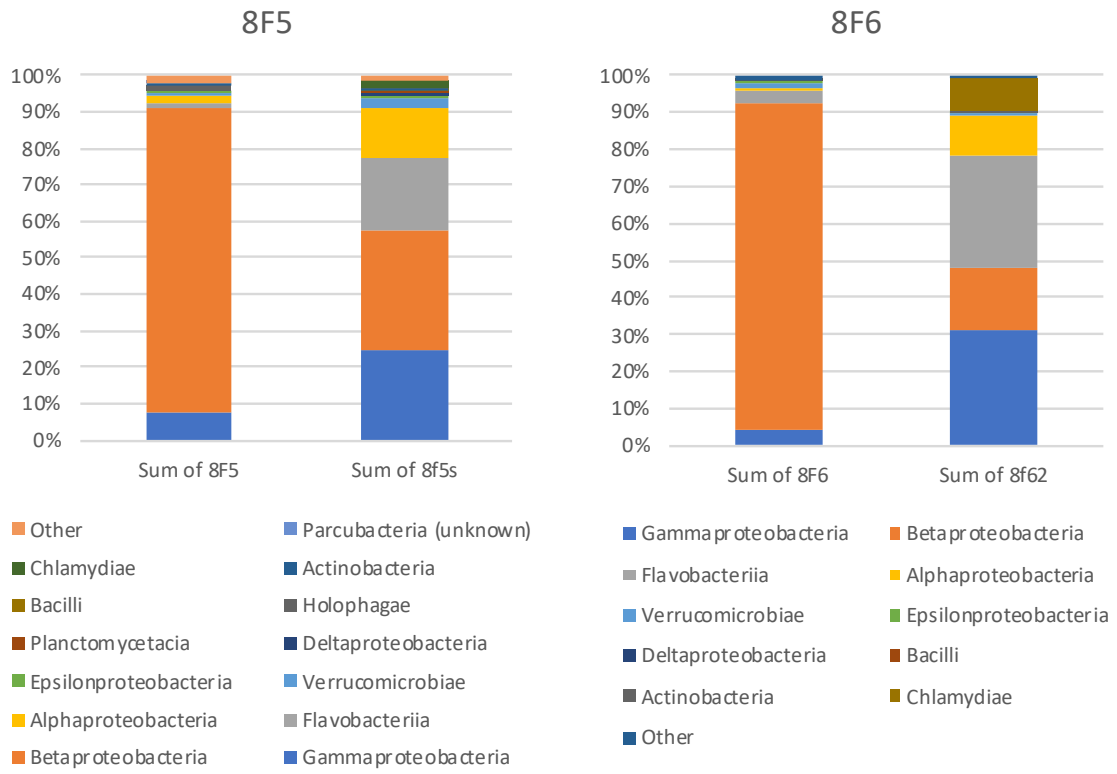
D.1 Hierarchical clustering of swabs and biopsies

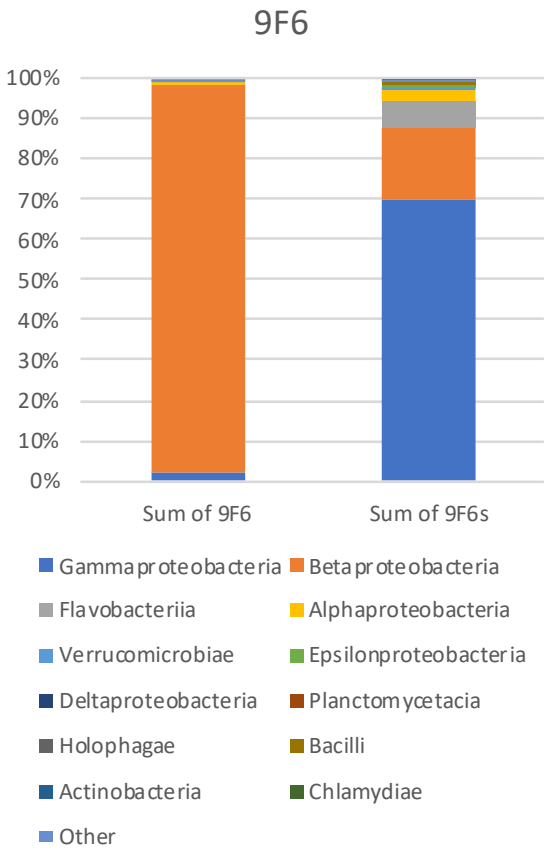


D.1: Identical to Figure 6.2 in the main thesis, this figure illustrates the histological findings of gills from which swabs and biopsies were obtained and demonstrates no significant differentiation of samples by gill disease.

D.2 Stacked bar plots

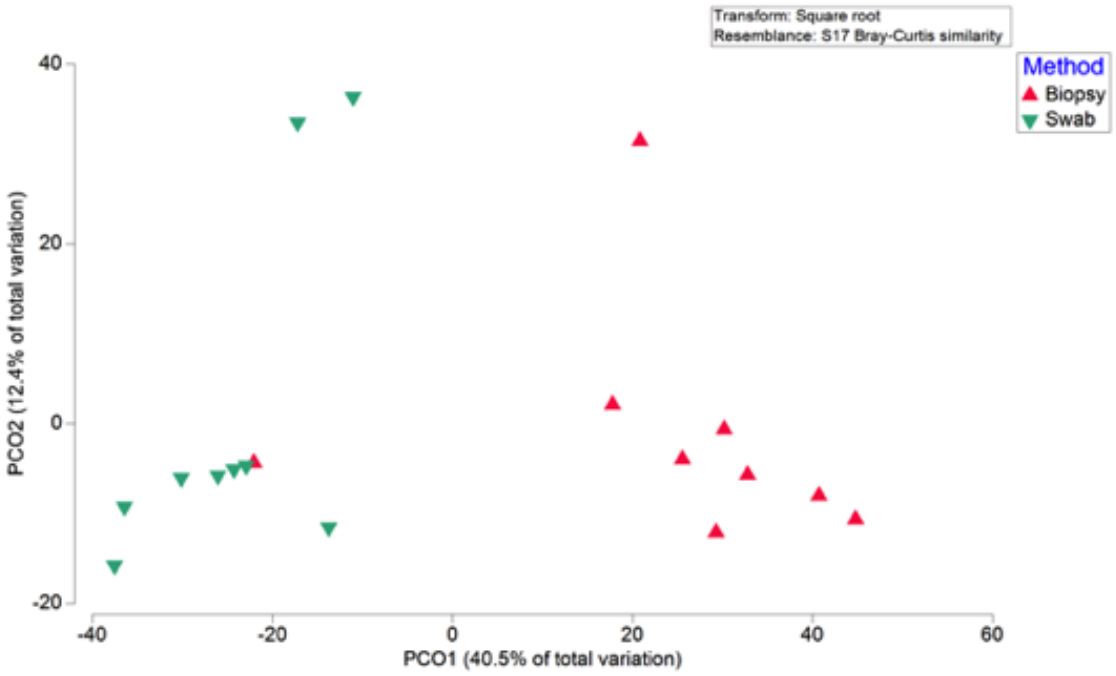


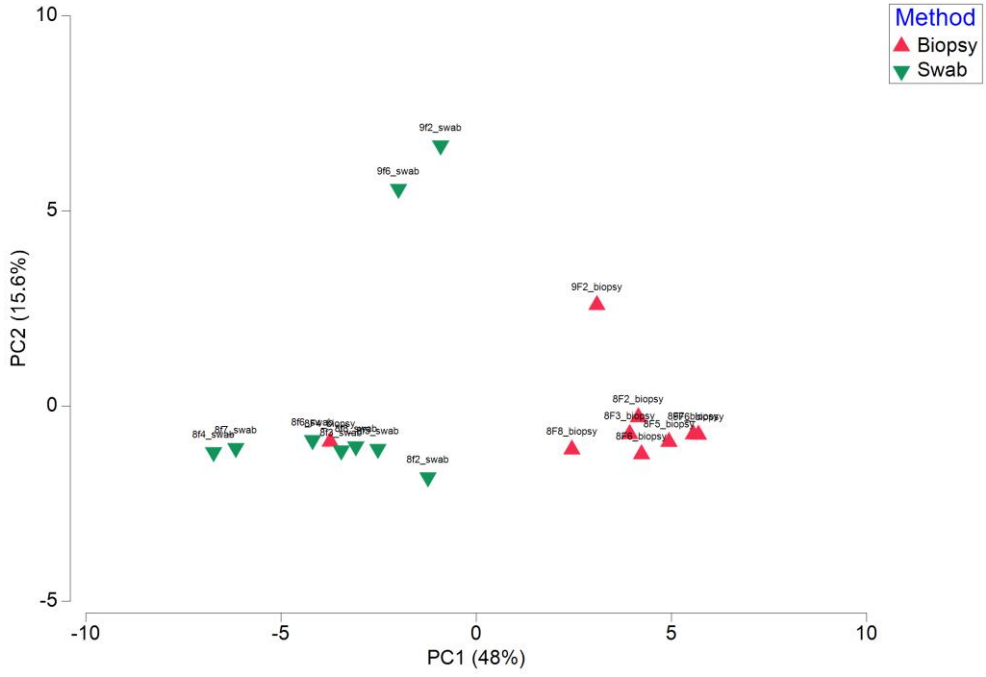




D.2: Remaining stacked bar plots from individual fish. Biopsy (left) and swab (right) derived results demonstrate noticeable differences in relative abundance of microbes, but apparent bias towards Betaproteobacteria, and potentially underestimation of Gammaproteobacteria

D.3 Swab vs Biopsy scatter principal component results





Appendix E; Ethics Approval Documentation

University of St Andrews

School of Biology Ethics Committee

15 December2016

Project Title:	Atlantic Salmon Gill Health: Alterations to gill tissue during the annual production cycle with specific focus on consequences of exposure to zooplankton
Researchers Name(s):	Morag Clinton, David Ferrier and Andrew Brierley
Supervisor(s):	Prof Andrew Brierley

Thank you for submitting your application which was considered by the Biology School Ethics Committee on the 15th December 2016. The following documents were reviewed:

1. Animal Ethics Form 24/10/2016

The School of Biology Ethics Committee approves this study from an ethical point of view.

Approval is given for five years. Projects, which have not commenced within two years of original approval, must be re-submitted to the School Ethics Committee.

You must inform the School Ethics Committee when the research has been completed. If you are unable to complete your research within the five year validation period, you will be required to write to the School Ethics Committee to request an extension or you will need to re-apply.

Any serious adverse events or significant change which occurs in connection with this study and/or which may alter its ethical consideration, must be reported immediately to the School Ethics Committee, and an Ethical Amendment Form submitted where appropriate.

Approval is given on the condition that local permits are obtained prior to the project starting, and also on the understanding that the ASAB Guidelines for the Treatment of Animals in Behavioural Research and Teaching published in Animal Behaviour, 2003, 65, 249-255, are adhered to.

Yours sincerely,

Convenor of the School Ethics Committee

Ccs School Ethics Committee
Dr Tamara Lawson (Home Office Liaison Officer)

SEC Convenor, CBD, Dyers Brae House, Greenside Place, St Andrews, Fife KY16 9TH, Scotland
Email: bioethics@st-andrews.ac.uk Tel: 01334 462054

Post-submission note

The following note is made subsequent to submission and acceptance of this thesis, but is pertinent for inclusion due to the impact on results contained therein.

Following communication with the industry partner of this project, the health team of SSF's has advised that hydrogen peroxide treatment (Paramove) of fish was not performed in isolation, but in combination with additional on-farm husbandry procedures. These additional treatments may have additionally influenced microbial communities, impacting the results and conclusions of Chapter 5.

As hydrogen peroxide treatments were not performed in isolation as was previously understood, the conclusions of this chapter must therefore be revised prior to submission of these results to any academic publication. The effected analysis (within section 5.4.5.1) was correct then based on the available information at the time of submission, however requires to be revised based on subsequent additional husbandry information. A revised analysis will include reference to the multiple factors that might have impacted adherent microbial communities in combination, rather than the singular factor of hydrogen peroxide treatment.

8.

8. References

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