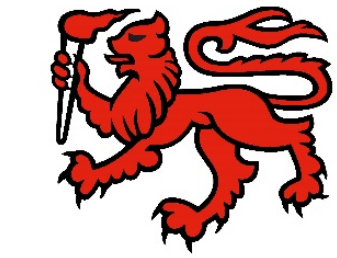

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The branchial microbiome and *Neoparamoeba perurans* infection

by

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Submitted in fulfilment of the requirements for the Doctor of Philosophy

University of Tasmania

May, 2022

University of Tasmania, July 2021

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The CSIRO QLD Animal Ethics Committee (AEC) in accordance with the "Australian code for the care and use of animals for the scientific purposes" approved the care and use of fish for this experiment (AEC approval numbers 2017-35, 2018-09 & 2018-18). The UTAS Animal Ethics Committee provided substantiation of these applications as external research activities (approval number A0017453)

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Statement of Co-Authorship

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PAPER 1: Located in Chapter 2

Slinger, J., Adams, M.B., Wynne, J.W., 2020. Comparison of bacterial diversity and distribution on the gills of Atlantic salmon (Salmo salar L.): an evaluation of sampling techniques. J. Appl. Microbiol. jam.14969. https://doi.org/10.1111/jam.14969

Author contributions:

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Analysed the data: Candidate, Author 1, Author 2

Contributed reagents/materials/analysis tools: Candidate, Author 1, Author 2

Wrote the manuscript: Candidate, Author 1, Author 2

PAPER 2: Located in Chapter 4

Slinger, J., Adams, M.B., Stratford, C.N., Rigby, M., Wynne, J.W., 2021. The Effect of Antimicrobial Treatment upon the Gill Bacteriome of Atlantic Salmon (*Salmo salar* L.) and Progression of Amoebic Gill Disease (AGD) *In Vivo*. Microorganisms 9, 987. https://doi.org/10.3390/microorganisms9050987

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PAPER 3: Located in Chapter 5

Slinger, J., Adams, M.B., Wynne, J.W., 2020. Bacteriomic Profiling of Branchial Lesions Induced by *Neoparamoeba perurans* Challenge Reveals Commensal Dysbiosis and an Association with *Tenacibaculum dicentrarchi* in AGD-affected Atlantic salmon (*Salmo salar* L.). Microorganisms 8, 1–17. https://doi.org/10.3390/microorganisms8081189

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Date:	16/07/2021	16/07/2021	19/07/2021

Conference papers/Conference proceedings

Slinger, J., Adams, M.B., Wynne, J.W. Amoebic gill disease (AGD): Does bacteria play a role?. In: 5th Symposium on Aquatic Animal Health and Biosecurity; 9-12 July 2019; Cairns, Australia. FRDC. http://hdl.handle.net/102.100.100/366114?index=1

Slinger, J., Adams, M.B., Wynne, J.W. Characterising the role of bacterial gill flora on amoebic gill disease (AGD). In: 1st International Symposium on Mucosal Health in Aquaculture; 9-11 September 2019; Oslo, Norway. Nofima. http://hdl.handle.net/102.100.100/366115?index=1

Abstract

Amoebae are unicellular protists distributed throughout terrestrial and aquatic environments. Commonly known as bacterivores or detritivores, members of the *Amoebozoa* group can parasitise higher vertebrate hosts and cause infectious disease. Furthermore, the virulence of such amoebic infections can in some cases be mediated by the presence of specific bacterial co-factors at the host-pathogen interface. Amoebic gill disease (AGD) remains one of the most significant diseases affecting the productivity of Atlantic salmon (*Salmo salar* L.) aquaculture, incurring significant costs to the Australian salmonid industry. The aetiological agent *Neoparamoeba perurans* is a free-living marine amoeba, which colonise gill mucosal surfaces eliciting often fatal branchialitis in affected fish. Although Koch's postulates have been established for AGD, *N. perurans* is a multi-organism complex of amoeba, a kinetoplastid endosymbiont and associated bacterial consortia. Determination of virulence factors that underpin AGD pathogenesis is therefore complicated by the potential interplay between these organisms. Additionally, commensal or pathogenic microbes that simultaneously colonise the host gill could potentially influence the course of AGD. Bacteria and *N. perurans* inhabit the same ecological niche, sharing resources and space on the gill surface, although the dynamics of microbial communities in the context of AGD remain largely unknown. It was hypothesised that the type and abundance of bacterial taxa present may ultimately affect amoebic-host interactions. Therefore, the aim of this thesis was to characterise the gill mucus community in the context of AGD pathogenesis.

To investigate whether non-culturable bacteria may influence the course of AGD, methods and bioinformatic pipelines to accurately profile branchial bacterial communities required initial refinement and validation. Chapter 2 compared sampling techniques and preservatives and tissue collection strategies. Results indicated that non-terminal mucus swabbing of the gill surface provided a robust bacteriomic representation of whole gill filaments. This study also demonstrated that the bacterial communities across different gill arches were not homogenous, and that both the diversity and richness of these communities upon the posterior holobranch were significantly decreased. Development of a suitable method to effectively reduce gill bacterial loads was required to manipulate the bacteriome prior to AGD challenge. Chapter 3 used antimicrobial treatments to reduce the bacterial gill load, and assess subsequent community change over a two-week timeframe. Chemical therapeutant baths and orally administered antibiotic elicited a perturbation event characterised by a significant bacterial dysbiosis on the gill surface. The post-treatment impacts of antimicrobial usage resulted in large scale bacterial

imbalance, and promoted the proliferation of potentially pathogenic genera. A subsequent *in vivo* challenge trial (chapter 4) exposed antimicrobial treated fish gills to wild-type *N. perurans*, to identify changes in progression of AGD and examine community dynamics on the gill. Results indicated that AGD developed in amoebae exposed groups irrespective of antimicrobial treatment and subsequent duration of dysbiosis. In addition, infection load and disease signs were marginally more advanced in fish treated with chloramine-t following challenge with *N. perurans*. Furthermore, the bacterial community that developed with AGD onset was prevalent in known pathogenic taxa (including *Aliivibrio*, *Tenacibaculum* and *Pseudomonas*) which increased in abundance concurrent to AGD severity. Subsequently, chapter 5 investigated potential linkage of bacterial taxa and AGD affected gill tissue. Bacterial community profiling was applied to branchial lesions in contrast to adjunct, unaffected filaments. Diversity of gill lesion material was decreased significantly, and dominated by the Flavobacterium *Tenacibaculum dicentrarchi*. This bacterium was moderately correlated in abundance with *N. perurans* offering new insights on the association between *Tenacibaculum* and *N. perurans* during AGD progression.

Taken together, these studies provide an approach to reflect bacterial load and diversity present upon gill mucosal surfaces, and how AGD progression impacts this dynamic. We demonstrate how these communities can be modulated to gain insights into the interactive dynamics of *N. perurans* and branchial bacteria during the development of amoebic branchialitis. The research presented in this thesis advances our pathobiological perspective of AGD and informs future research that seeks to further elucidate microbial co-factors that underpin the pathogenesis of AGD.

Acknowledgments

The PhD process has been both an incredible journey and a rapid learning curve which I am extremely thankful to have been given the opportunity to complete. Like most PhD projects there is a large contingent of people in both the foreground and background that I must thank and appreciate for making this possible.

It no doubt starts with my supervisory team, Dr James Wynne and Dr Mark Adams. James, you have been a real catalyst for my career development well before this PhD. I am so grateful our paths crossed, and for the endless guidance and expertise provided along the way. Mark, your proficiency and balance as researcher and technical expert is truly inspirational to me, and was crucial to the results of this project. Needless to say, I have the utmost respect for you both, and I will always appreciate the time invested in helping me become a 'real' scientist. Thankyou so much for all the tutelage, mentoring, enthusiasm, logic-bound wisdom, (the odd fishing discussion), thorough reviews, and the answering of many, many questions! – I could not have asked for better role models.

I wish to acknowledge that this research was supported by an Australian Government Research Training Program (RTP) Scholarship. Large components of this work were also funded in-kind by Tassal Group Pty Ltd. Thanks to Dr Brad Evans, Dr Colin Johnston and the Tassal RD members for their input and support. To the CSIRO Aquaculture program, thank you for the provision of a stipend and for generous opex budget necessary for this field of research. I am also extremely appreciative for the opportunity I was afforded to transition to a part-time technical role in the program while simultaneously completing these studies. My thanks go out to Stuart Arnold in particular, I don't know how far I would have progressed through the early stages of this arrangement without your determination and encouragement. I was able to attend several scientific conferences during my candidature made possible by travel grants awarded by the CoSE/IMAS travel scheme and the Mucosal Health in Aquaculture conference coordinators (Nofima).

A good deal of this project involved specialist skills of which I had limited prior knowledge or understanding. The most prominent of these was microbial bioinformatics where I was lucky to have Dr Andrew Bissett on-call to provide expert guidance and advice. An honourable mention goes to my fellow #qiimechat colleague Dr Tansyn Noble. Thanks for all the assistance, effort and patience! I have learnt so much from you along the way, and I think it's safe to say we got

there in the end. Thanks to Chris Stratford and Bert Koster for the technical assistance, effort, and good old-fashioned banter. The Atlantic salmon health facility at Bribie Island has been such an all-consuming part of my work life since 2015, and it made it much easier to shift focus elsewhere knowing that such capable staff were maintaining it while I was otherwise occupied. In reality managing an Atlantic salmon R&D facility in the subtropics is quite the achievement and I am immensely proud of our team for it! Thanks also to the remaining members of our conglomerated salmon health team at various CSIRO locations (Dr Chloe English, Dr Richard Taylor, Natasha Botwright, Russell McCulloch, Dr Francisca Samsing, Dr Paula Lima, Petra Quezada and Dr Ben Maynard) for their continued encouragement, assistance and advice. Special mention to Dr Megan Rigby for playing a significant role in molecular lab analysis in chapter 4. Thanks also to the Admin staff of the University of Tasmania for welcoming me back to UTAS and always accommodating the needs of an interstate-based student. Also, to staff of the IMAS fish health laboratory in Launceston, particularly Dr Melanie Leef, my visits were infrequent but always worthwhile.

A PhD research project is a selfish endeavour in many ways, as it invariably requires a commitment to spend long hours and invest a large amount effort, often in isolation. I am thankful to have had a great support network to draw on and pick up the slack from the other things I may have let slip off the radar! Thanks to my parents, Chris and Deb, for always supporting my absolute obsession in aquatic science, and providing me ample opportunities to chase that career. Dad, in my mind you personify the term resilience - seeing how you have dealt with and overcome such significant health adversities has been an inspiration beyond what I can articulate, and taught me to never doubt what is possible with the right mental application. To my sister Louisa, thanks for being an amazing sounding board and for subjecting yourself to many incoherent stories full of weird words and various things that weren't working! The Ryan family, Kev and Mary, for being such enthusiastic supporters, always asking for progress updates and being genuinely interested in hearing about the findings of each component of work. Lastly to my wife Jess, thank you for sharing the ups and downs of this journey with me, and all the sacrifices, support, love and inspiration you provide. I am not sure I would have made it without you.

Explanatory notes regarding thesis structure

This thesis is composed of a series of discrete experimental trials presented as discrete data chapters. Each data chapter has undergone peer review and been published within international journals as a standalone manuscript, with the exception of chapter 3, which is in preparation for eventual submission. As a result of this, unavoidable textual overlap occurs within the introductory sections of data chapters relative to chapter 1 in this thesis, and within the Materials and Methods sections of the research chapters. The referencing style adopted in this thesis follows the style of *Aquaculture*, and a single composite bibliography has been generated to encompass the entire document.

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Chapter 1. General Introduction

1.1. Global production of Atlantic salmon

Atlantic salmon (*Salmon salar*) are a fish species belonging to the *Salmonidae* family native to watersheds flowing into the northern aspect of the Atlantic Ocean (McDowall, 1996). The species is highly prized as a recreational angling target and a highly regarded fish for consumption (Sedgwick, 1988; Stead and Laird, 2002). These desirable culinary traits led to the establishment of farming of the species to ensure a readily available product. A failed attempt to culture a related salmonid species, rainbow trout (*Oncorhynchus mykiss*), was documented in 1912 (Paisley et al., 2010) and a period of static progress ensued. The concept of salmonid farming in an experimental capacity was revisited in Norway during the 1950's and 1960's, with success for both rainbow trout and Atlantic salmon (Sedgwick, 1988). From rudimentary beginnings, Atlantic salmon aquaculture has developed to become an international industry with multinational involvement around the 1980's (Stead and Laird, 2002). In FAO (2020), the 2018 global production totalled approximately 2.4 million metric tonnes with a market value of ~$18 billion USD (FAO, 2019). The salmonid aquaculture industry in Norway is also the largest export aquaculture sector in the world (FAO, 2020). Aquaculture continues to play a key role in future animal protein supply and contributes a 46% share of total seafood production globally (FAO, 2020). Consequently, salmonid aquaculture heavily contributes to provision of seafood product to fill market demand, in lieu of increasingly constrained wild fisheries.

1.1.1. Atlantic salmon aquaculture in Australia

Importation of Atlantic salmon occurred via shipments of fertilised ova transited to Australia between 1864 and 1870 (McDowall, 1996). These fish were liberated into waterways in Tasmania and Victoria as an intended angling resource, but had little success. After the formation and success of seapen aquaculture of Atlantic salmon in the northern hemisphere during the 1960's, further shipments of fertilised ova originating from the River Phillip on the eastern coast of Canada occurred. Fish were initially held at the Gaden trout hatchery in New South Wales before being transported to Victoria and subsequently Tasmania. The species once again failed to establish as a wild population, but this cohort was also maintained as a captive hatchery stock from which the current Atlantic salmon aquaculture population in Australia were derived (Ward et al., 1994). Currently the species is reared in a number of government and private owned hatchery facilities with the primary purpose to provide smolt that are on-grown in seapens. From the mid 1980's Atlantic salmon aquaculture progressed in Tasmania after legislation allowing commercial sale of salmonids was implemented. Following principals adopted in the

northern hemisphere and advancements to technology and equipment, the industry began to rapidly grow and expand. Farmed Atlantic salmon in Tasmania contribute ~90% of total salmonid aquaculture in Australia, and are the highest value agriculture resource in the state (DPIPWE, 2018). Atlantic salmon account for the highest value and production tonnage of all aquaculture species in Australia (Steven et al., 2020), a value predicted to exceed AUD $1 billion by 2023 (Mobsby et al., 2021). An extensive selective breeding program has been active within Tasmania since 2004, to provide high quality stocks for commercial farming operations in the state, further securing the industry (Elliott and Kube, 2009). The breeding program objectives include promoting maximal growth rates, mitigating against early maturation and increasing resistance to disease (Elliott and Kube, 2009).

1.2. Fish immunity and gill function

A crucial aspect of finfish aquaculture is animal health, and the management and mitigation of poor health or disease to achieve predictable growth outcomes. Inherent stressors may arise with high-density culture facilitating the proliferation of infectious agents presenting substantial management risk (Noga, 2010). The response to pathogen infiltration is mediated by the immune system. In teleost fish this is constituted of both an innate and acquired (adaptive) response mechanisms (Watts et al., 2001). The innate immunity is primarily composed of a physical barrier (generally on mucosal surfaces such as the skin, gills and gut). This epithelial barrier is a living-active organ, which is capable of active transport in both directions and can retain and concentrate immune components close to the mucosae surface (Tort et al., 2003). It also provides exclusion to pathogen threats via production of antibacterial compounds including bacteriocins, antimicrobial peptides and hydrogen peroxide (Cabillon and Lazado, 2019). In the instance where the epithelial barrier is breached, an inflammatory response is provoked (Watts et al., 2001), in an attempt to lyse invading organisms. Often following successful pathogen infiltration, the specific (adaptive) immune system is activated. Adaptive immunity, or the non-self or allorecognition system then proceeds to identify pathogens via somatic mechanisms through production of B- and T-lymphocytes (Secombes and Wang, 2012). This system responds to antigens by mediation of different types of leukocyte cells, which are generated from a number of immune tissues including the thymus, head kidney, liver and spleen. Crucially, this sophisticated immune system provides protection against specific pathogens or antigens previously encountered.

A number of pathogens from bacterial, viral and protozoan origins are known to impact Atlantic salmon in both hatchery and seawater growout phases (Stead and Laird, 2002). Many of these potential pathogens infiltrate the fish at sites exposed to the external environment, including the

mucosal surface of the skin, gut and gill. The gill has a multi-faceted role and function in fish. The lamellar surface is an intricate and sensitive organ, which allows crucial gaseous exchange (O_2, CO_2), nutrient excretion (NH_3/NH_4^+), acid-base balance and osmoregulation of prominent ionoregulatory processes (Cl^-, K^+, Na^+) (Evans et al., 2005; Secombes and Wang, 2012). The primary physical barriers of the fish (i.e. skin, gills) play host to a viscous colloid mucus layer (Cabillon and Lazado, 2019; Lazado and Caipang, 2014; Xu et al., 2016) which contains numerous protective antimicrobial compounds including enzymes and peptides, and facilitates growth and colonisation of a commensal microbial community. Branchial microbiota are thought to provide protection to the host by maintaining a diverse range of taxa which provide protection against pathogenic invaders via competitive exclusion. These commensal organisms produce and secrete various antibacterial compounds including bacteriocins, siderophores, hydrogen peroxide and organic acids designed to impact and limit pathogen bacteria (Gomez et al., 2013; Trust, 1975; Ubeda and Pamer, 2012). These components represent a beneficial barrier in immune defence and can be reflective of the health status of the fish (Gomez et al., 2013).

1.3. Global emergence of amoebic gill disease

Amoebic Gill Disease (AGD) has had a significant impact on Atlantic salmon aquaculture initially in Tasmania and increasingly around the world over the last decade (Marcos-López and Rodger, 2020; Mitchell and Rodger, 2011; Nowak et al., 2014; Oldham et al., 2016). Discovery of AGD as a largescale disease issue occurred shortly after the inception of salmonid culture in Tasmania (Munday, 1986). Initially it was observed that fish stocked at full salinity sites succumbed to an outbreak of overt gill branchialitis with mortalities approaching ~10% per week from seapen cohorts (Foster and Percival, 1988; Munday, 1986). Fish appeared to be under respiratory distress, demonstrating inappetence and lethargy. Furthermore, adverse water quality parameters including high salinity and water temperatures appeared to trigger severe AGD episodes (Munday et al., 2001; Zilberg and Munday, 2000). Smaller fish recently transferred from freshwater hatcheries appeared to be affected more significantly, potentially due to osmotic stress and associated immune suppression (Roubal and Lester, 1989). Anecdotally it was noted that the proportion of fish in any given pen under normal rearing/treatment circumstances adversely affected by AGD declines as they grow larger, which has since been supported by tank based studies (Smith et al., 2022). Since the discovery of AGD, management resourcing has increased in both historical and emerging areas of salmonid production including North America, Norway, Spain, Ireland, Scotland, France, Chile, New Zealand and South Africa (Kent et al., 1988; Mouton et al., 2014; Nowak et al., 2014). AGD has proven to be a more prevalent and destructive disease in the Australian industry over time (Clark and Nowak, 1999), and has

increasingly impinged upon producers in Ireland, Scotland and Norway both under the nomenclature of both AGD and CGD (Boerlage et al., 2020; Powell et al., 2015; Steinum et al., 2009; Young et al., 2007). In Tasmania the costs associated with AGD are estimated to be around AUD $40 million p.a. (Nowak and Archibald, 2018), not inclusive of associated productivity loss from treatment and increased demand on infrastructure and the environment.

1.3.1. Aetiology of AGD

Initially, *Paramoeba pemaquidensis* was identified as a potential cause of gill disease (Munday, 1986) following isolation from the gills of affected fish and matching descriptions made by Page (1970). *Paramoeba* sp. was subsequently ascribed as the cause of AGD (Roubal et al., 1989), from the first reported cases in Tasmanian Atlantic salmon by Munday (1986). Around the same time it was reported that other fish species were impacted by the same agent, which caused mortalities in sea urchins in both Norway and Canada (Jones, 1985; Jones and Scheibling, 1985) as well as Coho salmon (*Oncorhynchus kisutch*) in Washington State (Kent et al., 1988). In terms of nomenclature, *P. pemaquidensis* was subsequently moved into the genus *Neoparamoeba* by Page (1987) due to the presence of hexagonal glycostyles and the lack of surface scales when compared to *Paramoeba eilhardi*. Subsequent studies of *Neoparamoeba* sp. (Dyková et al., 2000) revealed the presence of an endosymbiont species Perkinsela (Hollande, 1980), a key identifying feature of the genus. After this time the aetiological agent for AGD was ascribed as *Neoparamoeba* sp., and it was later demonstrated that *Neoparamoeba pemaquidensis* did not elicit AGD in naïve salmon in a challenge setting (Morrison et al., 2005). Isolation of *Neoparamoeba branchiphilia* and *Neoparamoeba aesturarina* from diseased fish raised the possibility that the two species may also play a role in AGD development (Dyková et al., 2005), although it was later determined *N. branchiphilia* were unable to elicit branchialitis *in vivo* (Vincent et al., 2007). Subsequently Young et al., (2007) developed species-specific oligonucleotide probes to identify and name a new species, *Neoparamoeba perurans,* which was visualised by in situ hybridisation (ISH) to exclusively colonise gill lesions. Confirmatory challenge studies after this point culminated in the fulfillment of Koch's postulates confirming *N. perurans* as the primary aetiological agent of AGD (Crosbie et al., 2012).

1.3.2. AGD pathogenesis and detection diagnostics

During the onset of AGD, *N. perurans* trophozoites attach to the host gill surface eliciting an inflammatory response characterised grossly by the development of multifocal white-pale lesions on the gill lamellae (**Figure 1.1**; Bridle et al., 2006; Marcos-López and Rodger, 2020; Morrison et al., 2007, 2006b; Nowak et al., 2014; Pennacchi et al., 2016; Wynne et al., 2008).

Figure 1.1 Gross gill imagery depicting multifocal white-pale mucoid patches (lesions) which are indicative signs of AGD (photo courtesy of Dr Richard Taylor, CSIRO)

Pathologically, a marked proliferation of the respiratory mucosa can rapidly ensue trophozoite attachment. Proliferating lesions are characterised by epithelial hyperplasia, inflammatory infiltration and focal superficial necrosis of the branchial surface (Adams and Nowak, 2003; Morrison et al., 2006a; Munday et al., 1990a; Powell et al., 2008; Roubal et al., 1989; Young et al., 2008). Despite thorough documentation of the pathology of AGD, the virulence mechanisms employed by the pathogen in attachment and cellular degradation are not fully elucidated. The necrotic action of *N. perurans* has been postulated to involve cytolytic extracellular products (ECPs) (Bridle et al., 2015; Wiik-Nielsen et al., 2016). These compounds may directly destroy tissue, or degrade protective barriers and proteins within the host immune response, although this is not fully understood. Transcriptomic profiling of AGD-affected gills by Morrison et al. (2006a) demonstrated that a C-type lectin was upregulated at 5 days post infection (dpi) in AGD lesions, indicating that amoeba glycan epitopes may be able identifiable by the host. Recent research by Lima et al. (2021) has proposed that *N. perurans* is able to utilise surface glycan-binding proteins as a means of gill surface attachment, identifying mannobiose and N-acetylgalactosamine as potential candidates for gill surface binding. Recently a novel host-parasite interaction was described by Botwright et al. (2021) using a dual RNA-seq approach. Candidates

for virulence genes in *N. perurans* were identified, predominantly associated to invasion of host tissue, evasion of host defence and formation of mucoid lesions. This study noted that the transcription factors, *znfOZF-like* and *znf70-like* were significantly altered in Atlantic salmon under an AGD affected state. These results are promising but require further validation to confirm.

Several key diagnostics are employed by commercial operators and researchers to detect or monitor AGD. In commercial farm settings the detection of AGD and timing of treatment is generally achieved via visual gill scoring methods which assess the incidence and coverage of raised mucoid lesions (Taylor et al., 2009). Whilst essential as a rapid assessment tool commercially, this technique is presumptive and limited in terms of being able to differentiate similar non-AGD gill pathologies (Adams et al., 2004). Wet preparations of gill mucus and subsequent microscopic analysis is used as a more rigorous non-specific methodology (Zilberg and Munday, 2000). Diagnosis via morphology of the amoebae cells is a more time-consuming process and has limitations regarding high throughput of samples. Branchial histology is the most critical diagnostic technique for characterisation of AGD to visualise lamellar pathology associated with the presence of paramoeboid species (Adams et al., 2004; Adams and Nowak, 2001). Species-specific *in situ* hybridisation (ISH) probes, e.g. (Young et al., 2007) are also used in conjunction with gill histopathology to visualise the species with accuracy. After designation of the causal agent, highly sensitive DNA and RNA based qPCR assays were developed as a non-destructive diagnostic, and are regularly used to quantify 18S rRNA gene copies of *N. perurans* (Bridle et al., 2010; Downes et al., 2017).

1.3.3. Factors affecting AGD onset

The onset and progression of AGD has a strong seasonal influence with severity increasing during the summer period (Clark and Nowak, 1999). Several abiotic and biotic factors have been attributed to increased host susceptibility to AGD in salmonid culture. The most critical of these include water quality parameters such as increasing salinity (Munday et al., 1990b), which provides favourable conditions for amoebae to proliferate. Increased temperature (Benedicenti et al., 2019) and decreased dissolved oxygen are associated with increased severity of AGD (Oldham et al., 2020), but are not deemed to be direct causal factors for AGD onset as yet. Deteriorating water quality also leads to potential acute and chronic stress, which in turn lowers immune vitality and resilience. Further to this it has been demonstrated that aerobic respiratory capacity of AGD affected salmon was greatly decreased (Hvas et al., 2017). Other physical insults or damage to the gill (e.g. manual/mechanical handling of fish, jellyfish stinging cells (nematocysts), harmful algae or bacterial necrosis) increase stress to the animal and have been

identified as potential confounding factors in AGD. However no studies have supplied direct evidence exists to suggest that any of these factors exacerbate AGD directly (Adams et al., 2009; Powell et al., 2005a).

Reservoirs and vectors of *N. perurans* in proximity to the seapen environment have been investigated previously. *Neoparamoeba* spp. were isolated from biofouling surfaces on net material (Tan et al., 2002). Various species of *Neoparamoeba* have been isolated from marine sediment samples in the proximity of sea pens including *N. pemaquidensis* and *N. branchiphila* (Crosbie et al., 2002; Dyková et al., 2005), although a study by Hellebø et al. (2017) concluded that populations of *N. perurans* were not harboured within marine sediments in both recent and historical salmon production sites. Non-salmonid species which frequent lease areas were examined as a vector source, and concluded to be a negligible risk (Douglas-Helders et al., 2002), besides specific incidences where these species (or dedicated cleaner fish species) were implicated within the culture system with AGD pathology confirmed (Adams et al., 2008; Karlsbakk et al., 2013). Interestingly, very few of these postulated factors have been validated in experimental trials, and rely on anecdotal observations from commercial settings.

1.3.4. Treatment strategies for AGD

Fish affected by AGD become listless, often with flared opercula and increased buccal pumping (Taylor et al., 2021b). Subsequent latter stages of this respiratory distress leads to lethargy, hypernatremia and inappetence, with mortality often following if fish are not treated for the condition (Munday et al., 2001; Zilberg and Munday, 2000). Initial observations of AGD affected Atlantic salmon occurred in smolt recently transferred to seawater (Foster and Percival, 1988). Subsequent treatment responses to AGD onset initially involved moving fish back to inshore brackish water, but quickly developed into full immersion bathing in freshwater. This treatment is still considered current best-practice in Tasmania, but is an expensive undertaking in terms of time and resources (Clark and Nowak, 1999; Powell et al., 2015; Taylor et al., 2021b). Variants to traditional freshwater bathing have been thoroughly investigated. A number of chemical additives including hydrogen peroxide, chloramine-t and bithionol have been considered to either enhance the existing freshwater bathing efficacy, or as an *in situ* treatment in saltwater (Adams et al., 2012; Findlay et al., 2000; Florent et al., 2007a; Harris et al., 2005; Wynne et al., 2020a). Similar treatment with hydrogen peroxide is commonly used for controlling sealice (*Lepeophtheirus salmonis* & *Caligus* sp.) in the northern hemisphere (Powell et al., 2015; Vera and Migaud, 2016). Another potential treatment method is the use of antimicrobial or antiparasitic compounds administered orally to stock through feed (Florent et al., 2007b), or immunostimulant diets to offer protection (Dick, 2012; Mullins et al., 2020). While most of these

treatment variants show some potential for increased efficacy in amoebicidal action or trophozoite detachment rates, none have yet been employed at commercial scale in Tasmania. In most cases the identified improvement does not offset the risk of adverse stock reactions to incorrect administration. The use of both traditional freshwater bathing and other derivative treatments are likely to induce a broader antimicrobial action, impacting a wide range of microbial constituents, including commensal bacteria (Inglis, 2000; Lokesh and Kiron, 2016; Sekkin and Kum, 2011)

1.4. Microbial ecology and microbial community profiling

The commensal microbiome is the collection of genetic material from resident and transient microbes (inc. bacteria, fungi, viruses and archaea) which in turn make up a microbial community. Despite colonising external surfaces, this microbial community is often conceptualised as a living organ of the host in higher vertebrates, such is the impact and role it plays in biological function (Baquero and Nombela, 2012). The intrinsic role that the bacterial component of the microbiome (commonly defined as the bacteriome) play is best illustrated in examples where the absence of such microbes can be detrimental. For example, significant work has been completed involving the germ-free culture of rodent species such as mice and hamsters, proving that the presence of microbiota definitively affects key components such as brain chemistry, nutrition, immune function (Phillips et al., 1955; René Dubos, 1966). Mucosal microbiota play a significant role in many essential immunologic processes, along with hormonal and metabolic homeostasis (Antwis et al., 2017; Francino, 2016; Merrifield and Rodiles, 2015; Mohammed and Arias, 2015). Similarly, certain disturbances or perturbations of such communities can lead to imbalance, with detrimental effects often manifesting as disease outbreaks (Egan and Gardiner, 2016).

1.4.1. Antimicrobial activity and effect on mucosal microbiota

The incidence of disease outbreak is common in aquaculture production systems globally where production systems are stocked with fish at high density (Austin and Austin, 2016). From a management and stock perspective, antimicrobial treatment is often essential to maintain animal welfare and productivity (de Bruijn et al., 2018; Noga, 2010). Most common antimicrobial treatments used in aquaculture are broad spectrum in nature, impacting upon many bacterial species. Treatment application usually aims to target a specific pathogen, but can inadvertently impact the host commensal bacteria depending on the agent administered. Oxytetracycline hydrochloride (OTC), for example, is a commonly used broad spectrum antibiotic which has a bacteriostatic mode of action, making it suitable to treat a range of aquatic bacterial infections (Ambili et al., 2013; Rodrigues et al., 2017). Commonly administered through either immersion

bath or in-feed methods, OTC is appropriate for low dose chronic exposures as a tool to reduce bacterial levels (Austin and Austin, 2016). Chloramine-t (Cl-T) is an oxidative chemical compound employed as a therapeutic bath treatment in finfish aquaculture. Cl-T is effective at lysing the cell walls of gram-negative bacterium and fungal cells, and is one of the most commonly used treatments for bacterial gill disease (*Flavobacterium* spp.) in salmonid species (Harris et al., 2005; Stead and Laird, 2002).

Impacts on commensal bacteria diversity and richness has been demonstrated in a range of aquatic species. For example, Atlantic salmon treated with in-feed OTC showed a dramatic reduction in diversity of intestinal bacteria (Navarrete et al., 2008). Both Rosado et al. (2019) and Legrand et al. (2020) demonstrated that the microbial perturbation of antibiotic usage can last upwards of 18 days. Triclosan is a chlorinated aromatic antiseptic often used in therapeutic treatment of fish. Exposure of this chemical in both zebra danio (*Danio rerio*) and fathead minnow (*Pimephales promelas*) revealed both a reduction in bacterial diversity, but also an increase in opportunistic taxa such as *Pseudomonas* (Gaulke et al., 2016; Narrowe et al., 2015). Other therapeutic drugs can play a role in commensal dysbiosis. For example, Minich et al. (2020) completed an assessment of gill bacteria in Southern bluefin tuna (*Thunnus maccoyii*) treated with the anti-parasitic, Praziquantel, revealing a significant reduction in diversity and richness.

1.4.2. Role of microbial species on the gill

Commensal gill microbiota are linked to the respiratory mucosal layer in fish (de Bruijn et al., 2018). The diverse bacterial community which colonise this space play a key role in the health and immune capacity of the host (Gomez et al., 2013). Commensal bacteria produce beneficial compounds including antimicrobial peptides, enzymes, bacteriocins and hydrogen peroxide species, as well as providing competitive exclusion to opportunist pathogens (Cabillon and Lazado, 2019; Merrifield and Rodiles, 2015). Perturbations such as stress, antibacterial treatment or infection can lead to disturbance or imbalance in this community, which leaves the gill vulnerable to several diseases or disorders. Water surrounding the fish gill plays host to a high diversity of potentially pathogenic species, which can prove infectious to the host organism. Compromise to the gill mucus barrier can allow pathogenic opportunists to infiltrate and cause infection. This process can detrimentally impact the composition and abundance of commensal microbial communities (Schmidt et al., 2016a). Termed 'dysbiosis', this imbalance in microbial communities can also directly contribute to a disease phenotype.

Stress originating from culture conditions often leads to an increasing incidence of bacterial outbreaks (Bowker et al., 2013; Henriksen et al., 2017). For example, a recent study by Mota et al. (2019) observed thinner epidermis in Atlantic salmon exposed to a CO_2 concentration greater

than 19 ppm in RAS systems, rendering the host more susceptible to pathogen infiltration. Acute cold-water stress applied to the late egg stage significantly affected the gut and skin community of larval Atlantic salmon (Uren Webster et al., 2020). Similarly, seawater transfer of Atlantic salmon smolt caused an appreciable transition of the microbiota occupying the skin mucus (Lokesh and Kiron, 2016). Disease outbreaks also have significant impact upon the commensal microbiota. Atlantic salmon affected by sea louse outbreaks showed a significant loss of bacterial richness and a destabilization of community composition (Llewellyn et al., 2017). Furunculosis (*Aeromonas salmonicida*) in largemouth bronze gudgeon (*Coreius guichenoti*) dominated the intestinal microbiota in affected individuals, exhibiting a significant dysbiosis compared to the unaffected community (Li et al., 2016). Evidence suggests that bacterial perturbations can also be linked to environmental factors.

In the northern hemisphere, salmonid culture is affected by several multi-microbe conditions including complex gill disease (CGD) (Gjessing et al., 2019; Herrero et al., 2018), which involves *N. perurans* among other mixed aetiological agents (Candidatus *Piscichlamydia salmonis*, *Desmozoon lepeophtherii*, salmon gill poxvirus and Candidatus *Branchiomonas cysticola*). Given that *N. perurans* can interact in concert with a mix of pathogenic microbes, further research into virulence factors for AGD is valid and warranted. Other conditions including proliferative gill inflammation (PGI) (Steinum et al., 2010) and proliferative gill disease (PGD) (Boerlage et al., 2020) are encompassed under the umbrella of complex gill disease, and further illustrate the nature of mixed-pathogen aetiologies and the immune response in gill tissues.

Many bacterial pathogens exist in both fresh and marine water which target infiltration onto the gill surface and pose a significant threat to fish including bacterial gill disease and Columnaris Disease (caused by *Flavobacterium* sp.), and Tenacibaculosis (*Tenacibaculum* sp.). These species are able to colonise more effectively under conditions where the host mucosal immunity is reduced (Avendaño-Herrera et al., 2006; Ostland et al., 1990; Powell et al., 2005b). Species including *Tenacibaculum* are known to produce cytolytic compounds called extracellular products (ECPs) (Avendaño-Herrera et al., 2006; Cano et al., 2019; Van Gelderen et al., 2009), which can assist in host cell degradation and inhibit defence mechanisms. The possibility remains that endosymbiotic or associated bacteria could provide such products for *N. perurans* to utilise upon gill attachment.

1.4.3. Evidence for amoebic-bacterial cofactors

Free-living amoebae exist in various environments including soil, air, dust, and aquatic habitats (Molmeret, 2005). Many species are ubiquitous and can perform a variety of roles within specific ecosystems. Amoebae have developed complex evolutionary relationships with bacteria species

throughout time, acting primarily as a predator, assisting in keeping microbial loads in balance (Winiecka-Krusnell and Linder, 2001). Bacteria are a key driver for many biological system processes and functions (Molmeret, 2005), and have evolved many strategies to coexist with amoebae in the same niche. Symbiotic relationships between amoebae and bacteria exist, ranging from antagonistic to mutualistic (Shi et al., 2021). The social amoeba *Dictyostelium discoideum* predates on a range of bacteria, with selective predation playing a role in the bacterial community structure (Rosenberg et al., 2009). Recognition of bacterial prey sources is achieved via chemotaxis and phagocytosis (Shi et al., 2021). This process allows a pathway for amoebae-resistant bacteria (ARB) to parasitise amoebae as a transient host (Tosetti et al., 2014), bypassing digestion and harbouring themselves within trophozoites. Once located within the intracellular environment, some bacteria can replicate and exit the cell or utilise nutrients to proliferate within the organelles or cytoplasm (Strassmann and Shu, 2017). Phagocytosed bacterial taxa including enteropathogenic strains of *Escherichia coli* augment the cytolytic effects of *Entamoeba histolytica* trophozoites and increase virulence in amoebiasis (Galván-Moroyoqui et al., 2008). Similarly, it has been demonstrated that *Legionella* spp. is capable of passaging through *Acanthamoeba castellanii* and *A. astronyxis* with improved resilience to adverse conditions and increased invasiveness toward epithelial cells (Boamah et al., 2017; Guimaraes et al., 2016). Such examples demonstrate that specific bacterial taxa or bacterial loads can impact on how a given amoebae species interacts with its environment.

Electron microscopy has previously demonstrated that intracellular bacteria was located in trophozoites from aquatic amoebae including *Acanthamoeba*, *Nuclearia, Thecamoeba* and *Neoparamoeba* (which also contain a eukaryotic endosymbiont) (Dyková and Lom, 2004). Findings such as these provide a basis for the suggestion that intracellular bacteria may provide beneficial compounds such as an enzymes or proteinaceous agents which contributes to the pathogenic potential of the organism. To examine this further in the context of AGD, the relationship of bacterial communities during progression requires clarification. Until the fulfilment of Kochs' postulates (Crosbie et al., 2012), sustainable *in vitro* culture of *N. perurans* was problematic and subsequent re-infection of naïve hosts was not successful. This complication arose from a deemed loss of virulence, where naïve salmon challenged with cultured *N. perurans* trophozoites did not express clinical signs of AGD (Bridle et al., 2015). Historically the *in vitro* inoculation process has often required use of antibacterial agents to mitigate against bacterial overgrowth in the culture plate environment (Kent et al., 1988; Morrison et al., 2005; Vincent et al., 2007). Although many of these scenarios included *in vitro* cultures of other *Neoparamoebae* species (such as *N. pemaquidensis*) which did not elicit AGD, the premise remains that microbial modulation in

the culture flask environment may impact function of pathogenic amoebae. *In vitro* attenuation was previously described in *Acanthamoeba* sp., where a loss of virulence occurred after prolonged axenic culture, however once passaged through the host (rat) organism, infectivity was regained (Veríssimo et al., 2013). It has also been documented that *in vitro* culture in the absence of *Escherichia coli* reduced virulence of *Entamoeba histolytica* (Wittner and Rosenbaum, 1970) the causal agent of liver abscess in humans. It was reported that extended *in vitro* culture of *Naegleria fowleri*, an amoebae that causes meningoencephalitis, lead to an observed reduction, and eventually loss of virulence (Wong et al., 1977).

Examples of bacterial-amoebic interactions also extend to *in vivo* scenarios. In humans, amoebiasis is a serious health condition affecting patients across the world (Mirelman, 1987). The causal agent of this condition is *Entamoeba histolytica*, which is free living and can harmlessly colonise the lumen of human intestine under normal conditions. However, *E. histolytica* trophozoites can become pathogenic, causing severe dysentery when other microbial species are present yet non-virulent when cultured axenically. Infectivity returns once a microbial additive is presented, or the amoebae is passaged through the host and re-exposed to gut microbiota (Mirelman, 1987). Keratitis of the human eye caused by *Acanthamoeba* species have been strongly linked to several bacterial co-factors (Badenoch et al., 1990). Exposure of the corneal epithelium to *Acanthamoeba spp.* trophozoites leads to radial keratoneuritis, stromal infiltrate and severe blindness. It is believed that contact lens usage serves as a key factor in transmission of trophozoites leading to keratitis (Garate et al., 2006; Schuster, 2002). Whilst it has been known as a low risk, high severity condition, a recent study by Neelam and Niederkorn (2017) proposed that the bacterial flora of the eye may play a crucial role in the onset of keratitis. The study results showed that the presence of *Corynebacterium xerosis* led to a significant increase in trophozoite attachment and *in vivo* pathogenicity in a rodent model scenario.

A potential cofactor in the pathogenesis of AGD may be the adjunct microbe community associated with the gill surface and /or amoebae trophozoites. The hypothesis being that since amoebae utilise bacteria as a feed source, bacterial taxa may interact or be implicated in the condition (Horn and Wagner, 2004). Such biological interactions could, in theory, alter the rate of AGD development or the severity of the condition. Microbiota associated with gill tissue and/or amoebae trophozoites may have the ability to influence the virulence of *N. perurans*. Previous studies have demonstrated specific bacterial taxa predominately associated with AGD affected fish, and that bacteria can be detected simultaneously in histopathology samples (Adams and Nowak, 2004). For example, bacteria that were identified as *Pseudomonads* were observed within and around *N. perurans* trophozoites (Roubal et al., 1989) on the gill interface. Further

studies observed that gill bacteria in AGD affected fish from both laboratory and field sampling were dominated by a phylotype assigned to *Psychroserpens*, proposing this species as an associated opportunistic pathogen in AGD (Bowman and Nowak, 2004). A series of culture-dependent studies were later carried out to determine the role of culturable bacteria in AGD (Embar-Gopinath, 2006). The genera *Winogradskyella* and *Staphylococcus* were found in association with AGD affected Atlantic salmon (Embar-Gopinath, 2006), proposing an implication in the development of AGD or linked to the presence of the condition. Following this, an *in vivo* challenge with *N. perurans* alongside cultured *Winogradskyella* resulted in higher percentages of gill filaments with gill lesions attributable to AGD (Embar-Gopinath et al., 2005a), demonstrating that altered bacterial loads or species may exacerbate AGD progression. Disease outbreaks involving AGD such as an event in Korea (Kim et al., 2017) showed that fish affected by *N. perurans* were also exposed to high levels of pathogenic bacteria including *Vibrio* sp. Interestingly, a recent study by MacPhail et al. (2021) sequenced the bacterial profile of *N. perurans* cultures, deducing that *Vibrio* sp. were highly abundant around and within the trophozoite cytoplasm. Such linkages provide further information towards understanding the effects of the microbial consortia associated with AGD.

1.5. Thesis aims and outline

The fundamental project aim underpinning this thesis was to investigate the effect of gill microbiota modulation on AGD onset and progression in Atlantic salmon. This PhD extends on previous studies in this field by combining functional studies to modulate gill associated bacterial load for the purpose of assessing disease severity, and using a 16S rRNA amplicon sequencing approach to characterise the relationship between gill and amoebae associated microbiota and AGD pathogenesis. The overarching objective of this study was to access and describe the relationship between commensal/symbiotic microbial communities and the pathogenesis of AGD. This was addressed as per the following.

First a study (described in chapter 2) was conducted to assess the effect of sampling methodologies on the gill microbiome and then develop an efficient, non-destructive and repeatable sampling method that would be employed throughout subsequent experiments. Next, in order to investigate the effects of the gill microbiota on AGD, we evaluated the efficacy of different antimicrobial treatments in terms of reducing the bacterial load and microbial diversity (chapter 3). The most efficacious bactericidal treatments were then exposed to fish to provide reduction of the bacterial load preceding an AGD challenge experiment, with severity of AGD compared (chapter 4). Finally, specific bacterial taxa whose abundance was higher upon gill

lesions compared to adjacent unaffected tissues was further investigated using a combination of diagnostic approaches (chapter 5).

The research presented in this thesis has shed new light on the role that the gill microbiome may play in AGD development. The relevance of these findings in the context of existing literature is finally discussed in chapter 6, including potential future research avenues in this field.

Chapter 2. Comparison of bacterial diversity and distribution on the gills of Atlantic salmon (*Salmo salar* L.); an evaluation of sampling techniques

This chapter is a verbatim reproduction from the following published paper:
Slinger, J., Adams, M.B., Wynne, J.W., 2020. Comparison of bacterial diversity and distribution on the gills of Atlantic salmon (*Salmo salar* L.): an evaluation of sampling techniques. J. Appl. Microbiol. jam.14969. https://doi.org/10.1111/jam.14969

2.1. Abstract

This study assessed bacterial diversity and richness in mucus samples from the gills of Atlantic salmon in comparison to preserved or fixed gill filament tissues. We ascertained whether bacterial diversity and richness are homogenous upon different arches of the gill basket. Bacterial communities contained within gill mucus were profiled using 16S rRNA gene sequencing. No significant difference in taxa richness, alpha (p>0.05) or beta diversity indices (p>0.05) were found between the bacterial communities of RNAlater preserved gill tissues and swab-bound mucus. A trend of lower richness and diversity indices were observed in bacterial communities from posterior hemibranchs.

Non-terminal swab sampling of gill mucus provides a robust representation of bacterial communities externally upon the gills. Bacterial communities from the fourth arch appeared to be the least representative overall.

The external mucosal barriers of teleost fish (e.g. gill surface) play a vital role as a primary defence line against infection. Whilst research effort on the role of microbial communities on health and immunity of aquaculture species continues, the collection and sampling processes to obtain these data require evaluation so methodologies are consistently applied across future studies that aim to evaluate the composition of branchial microbiomes.

2.2. Introduction

Health and vitality of the gill surface barrier has been identified as a key factor in production of finfish aquaculture species globally (Beck and Peatman, 2015). In species such as Atlantic salmon, the gill surface performs many functional roles including gas exchange, ion regulation, osmoregulation, acid-base homeostasis, excretion and pathogen defence (Evans et al., 2005; Secombes and Wang, 2012). Teleost fish gills are composed of four paired arches containing primary and secondary lamellae (commonly termed filaments and lamellae respectively) that underpin gill functionality (Hughes, 1984; Wilson and Laurent, 2002). The gill epithelium is thin and delicate (~6 μm in rainbow trout (*Oncorhynchus mykiss*), Secombes and Wang, 2012), has a large surface area (0.1-0.4 $m^2.kg^{-1}$ bodyweight) and is in constant contact with surrounding waters. This makes the gill interface a primary site for host-pathogen interactions to occur (Koppang et al., 2015).

The outermost layers of the gill are comprised of three distinct compartments. Firstly a mucosal layer which contains numerous protective antimicrobial enzymes and peptides for immune defence (Gomez et al., 2013; Xu et al., 2016). Secondly, a carbohydrate rich glycocalyx barrier, which overlies the respiratory mucosa. While there is distinction between these components, they function in concert due to their close association providing significant barrier function and protection from the external environment (Powell et al., 1994). The branchial mucus layer is constantly replenished, however loss of mucus or an epithelial breach can lead to respiratory compromise, pathogen infiltration and potential systemic infection (Lü et al., 2014). Commensal (resident and transient) taxa inhabit the interstitial boundary between the epithelium and the surrounding milieu (Llewellyn et al., 2014; Reverter et al., 2017). These commensal microbiota can competitively inhibit pathogenic taxa from accessing resources and colonising adhesion sites and causing inflammation (Naik et al., 2012, Okumura and Takeda, 2018).

While the dominant factors affecting gill colonisation by microbial communities in teleost fish include diet and environment (Webster et al., 2018), little information exists regarding changes in the microbiota community within specific locations or microhabitats on the gill. The gill assemblage is structurally complex consisting of eight holobranchs (or arches) each comprising anterior and posterior hemibranchs. Previous studies have identified potential differences in gill metabolic activity and blood flow related function (Hughes, 1966). Patterns of respiratory flow across gill arches was investigated by Paling (1968), using a marker parasite quantification (freshwater mussel *glochidia*) to determine the relative amount of flow across each arch pairing within the gill basket of brown trout (*Salmo trutta*). This study demonstrated that the relative flow volume over the 2nd and 3rd gill arch was higher than the 1st and 4th arch. This anatomical

design has the potential to influence the recruitment and retainment of bacterial taxa upon gill surfaces.

The magnitude of microbiomic gill studies is likely to increase substantially over the coming years given the global emergence of gill health issues affecting finfish aquaculture. A prominent example is branchial infection with the marine amoeba *Neoparamoeba perurans* which induces amoebic gill disease (AGD) in Atlantic salmon and other cultured fish species (Munday et al., 2001). Advancements in microbial community profiling have provided a greater insight into the bacterial taxa that occupy diverse environments (Jovel et al., 2016; Legrand et al., 2019). Rapid development of culture independent next generation sequencing (NGS) techniques have revolutionised the way bacterial communities can be studied and subsequently assessed (Egerton et al., 2018; Llewellyn et al., 2014). Gill microbiota has been previously studied in a number of teleost fish species, with typical sampling methodology including excision or biopsy of gill tissue, scraping of gill mucus, and swabbing of the gill surface layer (Bowman and Nowak, 2004; Minniti et al., 2017; Reverter et al., 2017; Slinger et al., 2020a). Given the structural and cellular complexity of fish gills, the sampling methodology employed may alter the output and consequently any interpretation commensurate to microbial abundance and diversity. Therefore, this study aimed to assess the diversity and composition of bacteria colonising interbranchial microhabitats and the inter-individual variance in a single cohort using both terminal and non-terminal sampling techniques. We provide a summary of the microbial recruitment obtained via distinct sampling techniques and demonstrate the appropriateness of mucosal swab sampling for subsequent microbiomic gill studies.

2.3. Materials and methods

All animal procedures were approved under application (#2018-9) by the Queensland CSIRO Animal Ethics Committee under the guidelines of the Australian Code of Practice.

2.3.1. Fish source and husbandry

Fish were obtained from a commercial fish hatchery in Tasmania, before being shipped (8 g mean weight) to the Bribie Island Research Center (Woorim, QLD) and grown in freshwater recirculation systems for approximately 7 months, undergoing regular monitoring and maintenance including size grading. Salmon parr were prepared for seawater transfer by exposure to 24 h photoperiod regimes (3600 lumen) for a period of 5 weeks before transitioning from fresh to marine water. Following preparation for seawater transfer, the fish (n=550) had an average weight of 218g (± 1.2) and were maintained in a 5000 L tank at a temperature of 14°C±0.5°C, dissolved oxygen 90-100% saturation, total ammonia nitrogen (TA-N) <0.5 mg-L, pH 7.8±0.1, and a salinity of 35±1 ppt. Fish were acclimated for a period of 21 days, and visually

assessed for signs of normal behaviour including feeding. Prior to sampling, all fish used were humanely killed using 100 mg L^{-1} of Aqui-S anaesthetic (Aqui-S NZ Ltd, Lower Hutt, New Zealand). This study utilised samples (both naïve and AGD affected) derived from a previous in vivo AGD experimental trial, as described below from Wynne et al. (2020).

2.3.2. Experimental challenge with *Neoparamoeba perurans*

After a habituation period (7 d) in seawater, 50 Atlantic salmon smolt (naïve to AGD) were transferred to a 1000 L seawater (35 ppt) flow-through tank with temperature maintained at 15±0.5°C, pH at 7.8±0.1 and dissolved oxygen maintained between 90-100% saturation. Fish were fed daily to satiation using Nutra 3 mm pellet (Skretting P/L, Cambridge, Australia). Habituation after handling was assessed visually by fish behaviour and feeding activity, this was conducted by staff highly experienced in salmonid husbandry. Remaining fish (n=500) were purposed for use in an AGD challenge trial (Wynne et al., 2020a), and were exposed to wild-type gill associated trophozoites of *Neoparamoeba perurans* as follows. Firstly, a sufficient volume of water (1 L) was taken from a recirculating system containing AGD affected Atlantic salmon, and the concentration of amoebae trophozoites was determined by centrifugation at 4000 g to concentrate cells into a final volume of 10 ml. Wild-type *N. perurans* stock concentration was then calculated using the mean of repeated trophozoite counts (n=10) via a haemocytometer. Once the density of infective amoebae cells was calculated, a sufficient volume of stock water was then introduced to the fish holding tank to achieve a final concentration of 100 *N. perurans*.L^{-1} (1 h static exposure). A smaller subset of 33 salmon were taken from the exposure tank and stocked into a single 500 L flow-through seawater tank (stocking density ~14.2 $kg.m^{3}$). Fish were subsequently maintained at 15±0.5°C, pH 7.8±0.1, 35 ppt and dissolved oxygen saturation between 90-100%. The infected fish were fed daily to satiation with a commercial diet (Nutra 3 mm, Skretting P/L, Cambridge, Australia) until sampling at 21 days post-infection (dpi).

2.3.3. Comparison of bacterial communities between mucus, preserved gill filaments and fixed gill filaments

To compare the microbial community between gill mucus and gill tissues (either preserved in RNAlater or fixed in seawater Davidson's solution) we sampled five AGD affected Atlantic salmon at 21 dpi (**Figure 2.1**). For consistency, only fish that were assessed at a gill score of 3 on the 6 point ordinal scale of AGD severity (as per Taylor et al., 2009) were selected for sampling in this study. Firstly, gill mucus was swabbed from the anterior hemibranchs (left side; LH1-LH4) by holding open the operculum and gently rolling a sterile cotton swab (Westlab) with three rotations over the entire length of the anterior hemibranch. The swab was then placed into a 1.5 ml tube containing RNAlater (1 ml). Two samples of gill filament tissue (approximately 0.5

cm2) were then excised using sterile micro-scissors from the dorsal region of the third holobranch on the right-hand side of the gill basket (RH3). One sample was placed into a 1.5 ml tube containing 1 ml RNAlater solution and the other sample placed into a 15 ml specimen container with 10 ml of seawater Davidson's fixative. RNALater preserved gill filaments and mucus swab samples were stored in the freezer at -20°C until DNA extraction. Samples which were stored in seawater Davidson's fixative were subsequently transferred to 70% ethanol 24 hrs after excision and stored at room temperature before DNA extraction.

2.3.4. Evaluation of bacterial communities across different gill arches

The bacterial communities between the four anterior hemibranchs on the left-hand side of the gill basket were compared in 10 Atlantic salmon smolt naïve to AGD (**Figure 2.1**). A sterile cotton swab (Westlab) was rolled along the arch surface (three rotations) of each hemibranch surface (LH1-LH4) before being placed into 1 ml of RNAlater and stored at -20°C prior to DNA extraction.

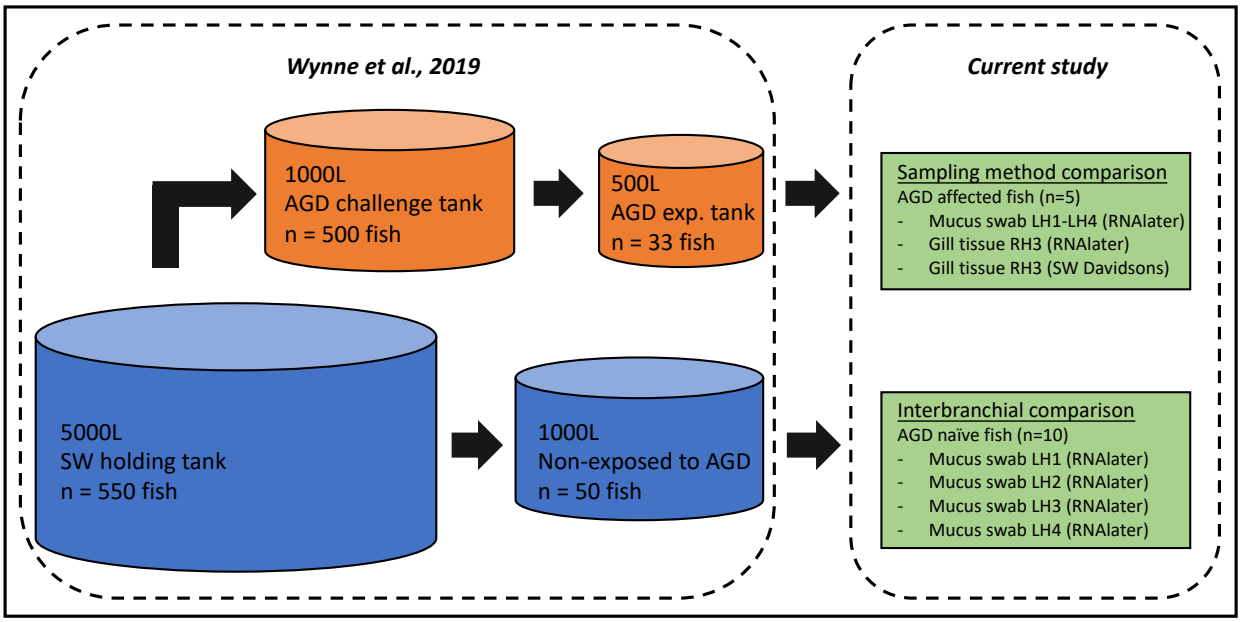

Figure 2.1. Schematic illustration of components of the experimental design conducted in Wynne et al. (2020), and the subsequent fish-source linkages to the current study. A total of 5 AGD affected fish were utilised for the sampling method comparison, while 10 naïve smolt were used for an interbranchial comparison.

2.3.5. DNA extraction and purification

Gill swab samples were subjected to agitation for 5 mins via a vortex genie (Bioline, London, UK), before samples were centrifuged at 17,000 g for 1 min to pellet all suspended material. RNAlater solution was carefully pipetted from each sample tube taking care to avoid the pellet. The total weight of excised gill tissues was standardised at 25 mg prior to DNA extraction (DNeasy Blood and Tissue Kit, Qiagen, Hilden, Germany), digestion (5 h), washing and elution.

Total genomic DNA was assessed for yield and quality using a Nanodrop ND-1000 spectrophotometer (Life Technologies, Carlsbad, US). Samples were stored at -80°C until sequencing.

2.3.6. 16S rRNA amplicon sequencing

Sequencing was performed at the University of New South Wales, Ramaciotti Center for Genomics (Sydney, Australia) via an Illumina miseq platform with 300 basepair reads from both the forward and reverse direction. The sequencing targeted the V1-V3 hypervariable region of the 16S rRNA gene in this study ("27F"AGRGTTTGATCMTGGCTCAG; and "519R"GWATTACCGCGGCKGCTG) as described by Lane et al., 1985 and Lane, 1991. A total of 33 cycles were performed with normalised DNA at an average of 10 $ng.\mu l^{-1}$. Samples were divided between two separate sequencing runs, with both runs including a mock positive control (ZymoBIOMICS Microbial Community Standard, Zymo Research), and two negative controls (blank swab process control and a blank DNA extraction laboratory control). Sequencing reads from the demultiplexed samples analysed in this study have been deposited in the NCBI Sequence Read Archive (SRA) under the BioProject accession PRJNA649054.

2.3.7. Bioinformatics pipeline

Raw Illumina amplicon sequencing data files were processed using the open-source software pipeline "Quantitative Insights into Microbial Ecology 2" QIIME2 (Caporaso et al., 2010). Paired end sequences from the forward and reverse reads were merged for each sample and were denoised using the q2-dada2 plugin (Callahan et al., 2016) with default parameters. Quality control including chimeric sequence removal from the dataset was completed during dada2 processing, along with subsequent removal of host DNA and exclusion of chloroplast and mitochondrial sequences. Amplicon Sequence Variants (ASV's) were classified taxonomically using the classify-sklearn method in the QIIME2 q2-feature-classifier plugin using default parameters (Bokulich et al., 2018). The SILVA 16S rRNA 99% taxonomy database release 132, (Quast et al., 2012), was used as reference sequences for taxonomic classification.

2.3.8. Statistical analysis

All statistics were performed in R version 3.6.0 (R Core Team, 2019). Samples were rarefied using R package QsRutils (Quensen, 2020) performed on a maximum subsampling depth of 5720 sequences per sample (**Figure S2.1**). Using the Phyloseq package (McMurdie and Holmes, 2013) taxonomic assignments were generated and alpha diversity indices calculated (Observed ASV's, Shannon diversity, Faith's phylogenetic distance). The alpha diversity metrics were analysed via non-parametric means (Kruskal-Wallis test) and further pairwise comparisons using a Wilcoxon Test (Rank Sum Test). Beta-diversity comparisons were made via NMDS using Bray

Curtis pairwise distances. Differences between groups was analysed using the Vegan package ANOSIM (Oksanen et al., 2018). Relative taxonomic abundance was analysed using a Kruskal-Wallis test and differential abundance testing between specific bacterial taxa was completed using the DeSeq2 package (Love et al., 2014). All figures were produced using the R package ggplot2 (Wickham, 2016).

2.4. Results

2.4.1. Challenge with Neoparamoeba perurans

Gross clinical signs of AGD including raised multifocal lesions on the gill surface were observed in AGD affected fish. During the challenge trial, conspecifics within the tank population sampled for this study were confirmed to be AGD affected via the presence of *N. perurans* and pathological changes characteristic of AGD were confirmed in affected fish by qPCR and histopathology (Wynne et al., 2020a).

2.4.2. Comparison of microbiota between mucus, preserved gill filaments and fixed gill filaments

Sequence data in this study averaged 40,569 raw sequence reads per sample and after removal of chimeric and mitochondrial/chloroplast associated sequences, a total of 1,350 unique ASV's derived from the 15 samples were identified.

Alpha diversity

Comparison of alpha diversity metrics were first performed between gill filaments collected by the three sampling methods; Gill Mucus Swab (GMS), RNALater Preserved Gill Filament (PGF), and seawater Davidson's Fixed Gill Filament (FGF). Observed ASV's were used to measure taxa richness, and Shannon and Faith's diversity indices to assess taxa diversity and diversity respectively. Samples from the FGF tissue had significantly lower observed ASV's in the FGF group (12.2 ± 1.1) when compared to both the GMS (61.8 ± 7.2) and PGF groups (75.8 ± 3.6) (**Figure 2.2A**; Wilcoxon, $p<0.001$). Samples from the FGF group also had significantly lower taxa diversity (1.79 ± 0.12) (Shannon diversity index; **Figure 2.2B**) and phylogenetic diversity (1.76 ± 0.10) (Faith's; **Figure 2.2C**) compared to the GMS and PGF tissues (Wilcoxon, $p<0.05$). RNAlater preserved tissue types were not significantly different from one another in all alpha diversity metrics. The PGF samples showed less inter-individual variation in alpha diversity metrics compared to the mucus samples with lower SEM between all three measured metrics.

Beta diversity

We assessed the bacterial beta diversity between the three distinct sample types taken from each fish. NMDS analyses based on Bray-Curtis distance (**Figure 2.2D**) indicated a group-level

difference when assessed (ANOSIM $p<0.05$). This is further evidenced as the NMDS plot shows clustering of samples from the GMS and PGF groups indicating a high degree of homogeneity in the bacterial community structure among these two sample types, whereas the FGF group clustered separately and was considered significantly different.

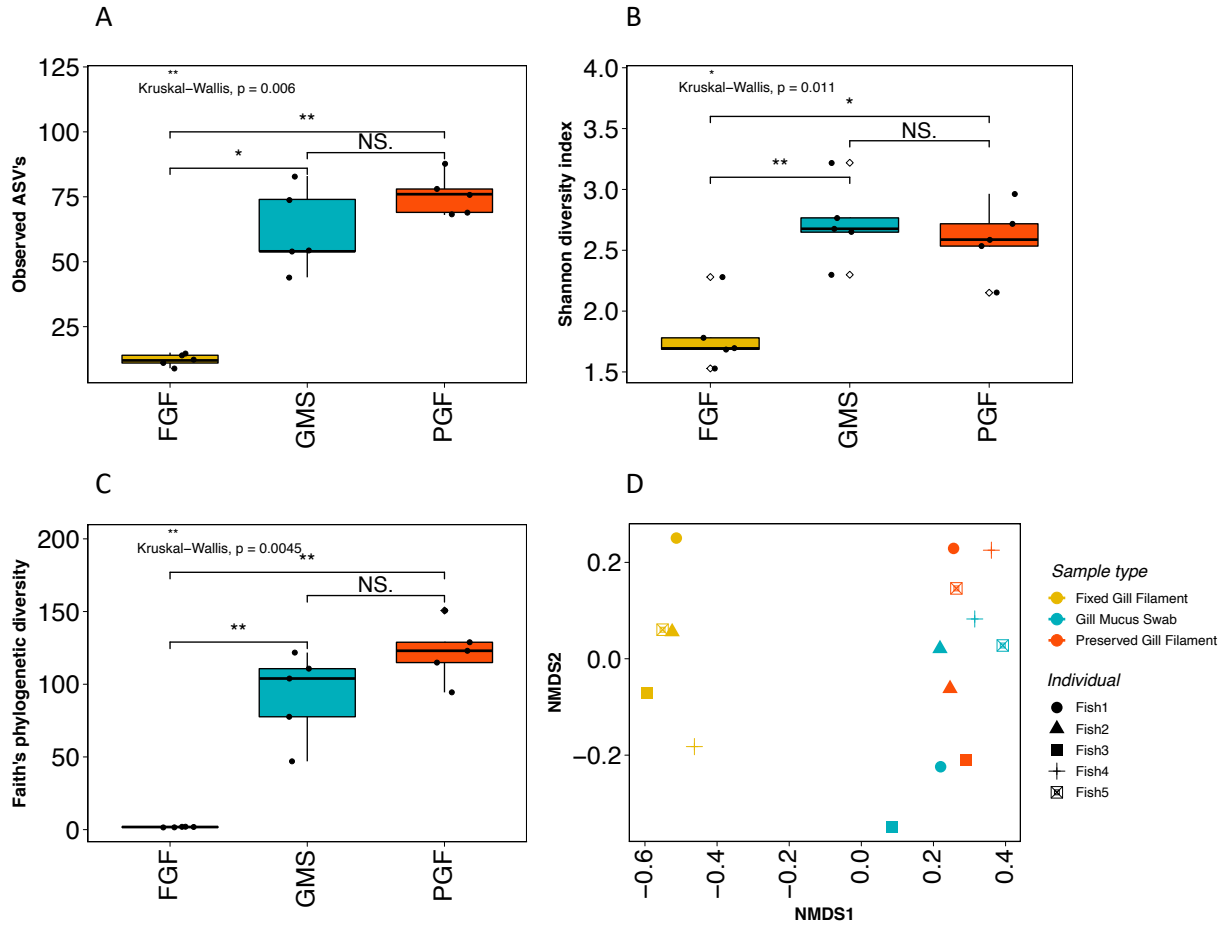

Figure 2.2. Alpha diversity metrics for community richness and diversity. Mean taxa richness (A) and diversity (B and C) were significantly lower in seawater Davidson's fixed gill filament (FGF) samples in comparison with gill mucus swab (GMS) and RNAlater preserved gill filaments (PGF). * denotes p value < 0.05, ** denotes p value < 0.001. Black dots represent each individual sample point, unfilled diamond shape indicates outliers. (D) - Bray Curtis NMDS similarity matrix comparing individual fish samples taken via three distinct sampling regimes. Colour difference indicates the sampling regime while shape denotes each individual fish sampled (each individual was sampled via three methods).

Taxonomic abundance

Samples analysed at the phylum classification show the microbial community was dominated by Proteobacteria, where abundance was largely homogenous between sampling types (43.9% - 64.1%; **Figure 2.3A**). One clear difference between the fish sampling groups was the total absence of *Verrucomicrobia* in the FGF (0%), with prevalent mean abundance in both GMS

(23.5%) and PGF (8.3%) samples. A much lower prevalence of *Bacteriodetes* (1.3%) in FGF was evident, along with an increase in *Actinobacteria* (31.3%).

Figure 2.3B shows differentially abundant taxa at the genus level within the three sample types (Kruskal-Wallis test). The FGF sample type plays host to a select group of prominent taxa including *Methyloversatilis*, *Pelomonas* and *Cutibacterium*. There is a clear absence of the Verrucomicrobia originating Rubritalea in this sample type, which is found in both the GMS and PGF sample types as mentioned above in **Figure 2.3A**. This assessment across differentially abundant genera between sample types demonstrates that some differences occur in bacterial recruitment based in the sampling regime (and therefore microhabitat targeted). GMS samples had significantly higher incidences of both *Rubritalea* and *Tenacibaculum* on the surface of the gill, while the taxa Pelomonas were generally underrepresented in the GMS while occurring in higher abundance in both tissue derived sample types.

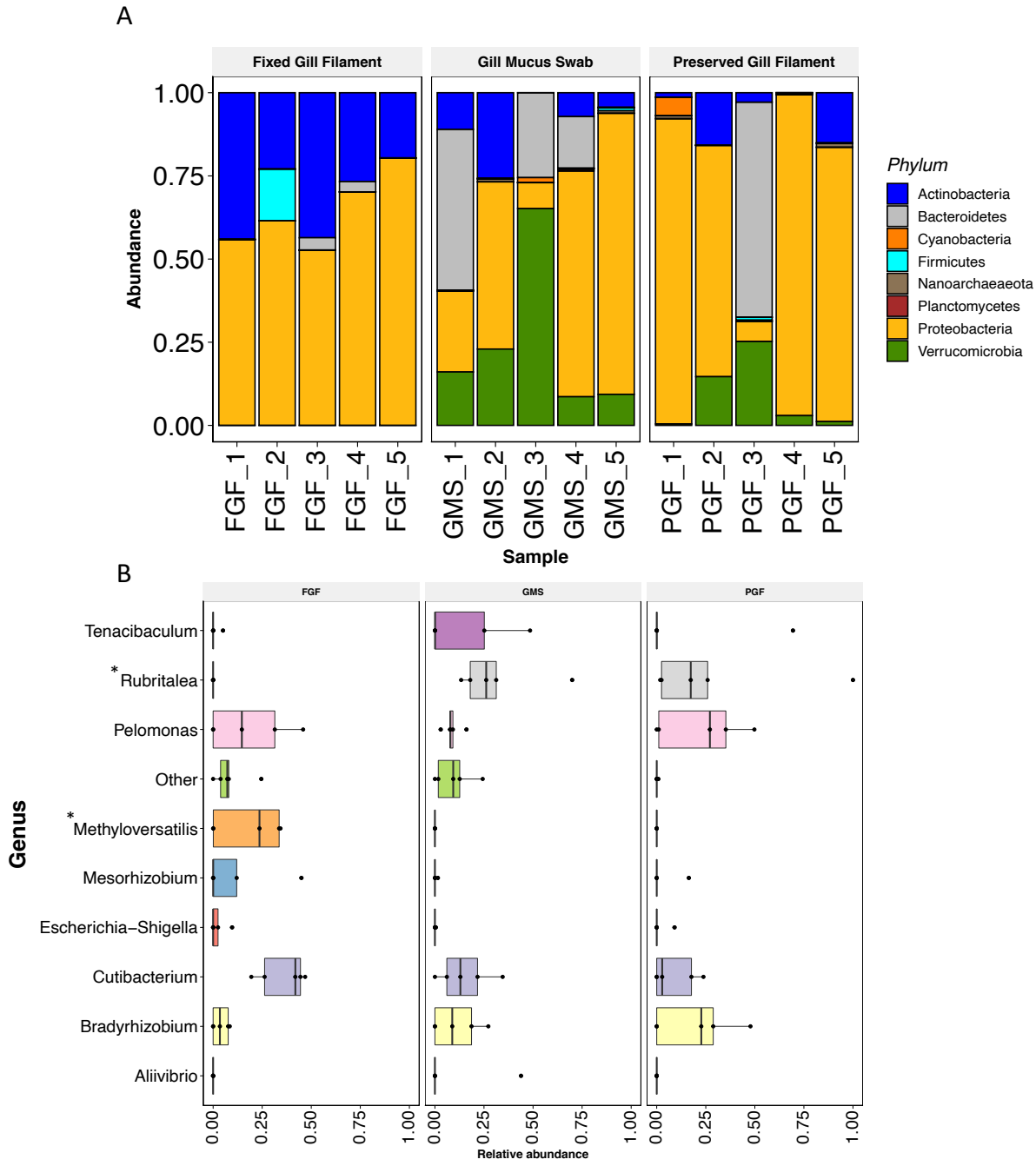

Figure 2.3. (A) Relative abundance of bacterial taxa classified at the phylum level, only taxa with > 1% abundance are shown. (B) Relative abundance plot showing genus level taxa considered significantly different between sampling type. * denotes significant taxa between hemibranch arches ($q < 0.05$). Black dots represent outlier points.

2.4.3. Evaluation of bacterial communities across gill arches

Next we compared the microbial community between gill hemibranchs. An average of 64,860 raw sequence reads per sample were generated, and after quality filtering (removal of chimeric and mitochondrial/chloroplast associated sequences), a total of of 922 unique ASV's from 40 samples were observed against the SILVA 99% taxonomic database (release 132).

Alpha diversity

The second component of this study compared the microbial diversity associated with interbranchial lamellae of individual fish within a cohort of AGD naïve salmon. A Kruskal-Wallis comparison of alpha diversity metrics was first performed between the four hemibranch arches of each fish (**Figure 2.4**). Analysis demonstrated that the observed ASV count was higher in the LH1 hemibranch (39.3 ± 4.5) and decreased towards LH4 (24.3 ± 1.9), with a subsequent significant difference between the first and fourth arch (**Figure 2.4A**; Wilcoxon, $p<0.05$). The taxa diversity (Shannon Index) also decreased from the LH1 (3.05 ± 0.09) to LH4 (2.70 ± 0.09) samples with the first and fourth hemibranch arches again significantly different (**Figure 2.4B**; Wilcoxon, $p<0.05$). Mean phylogenetic diversity was also lowest in the LH4 mucus swab samples, although this was not significant (**Figure 2.4C**; p=0.81).

Beta diversity

NMDS based on Bray-Curtis dissimilarities of bacterial profiles from different arches shows tight clustering of hemibranch positions across the fish cohort (**Figure 2.4D**). No significant effect of the hemibranch arch position and the bacterial community was observed (ANOSIM, p=0.224). Some inter-individual variation exists between fish samples, and in several individuals, there is a strong similarity between the first and second arch communities, although this was not observed consistently throughout the cohort.

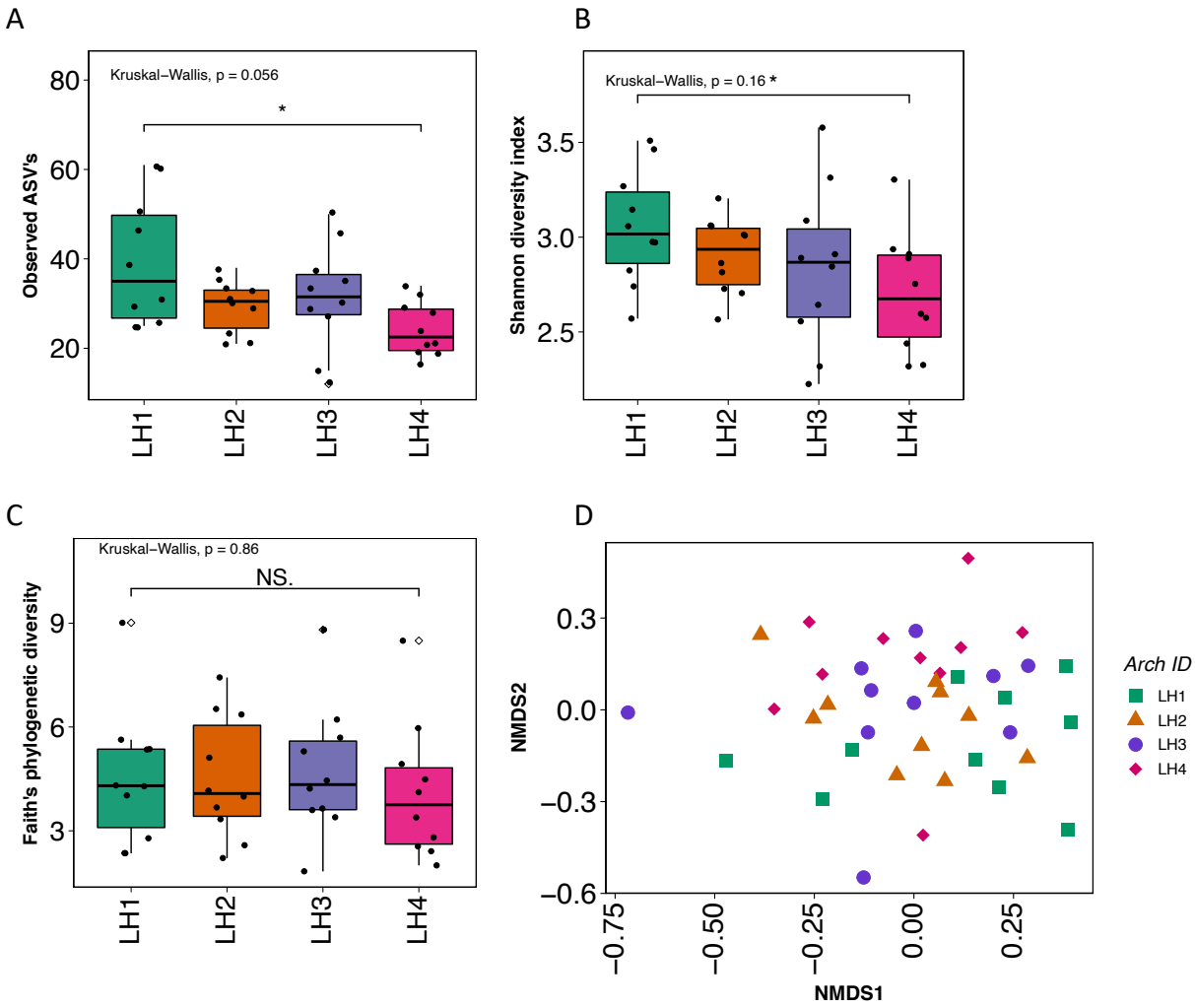

Figure 2.4. Diversity and richness of gill microbiota derived from interbranchial microhabitats. Mean taxa richness (A) and diversity (B and C) were significantly lower in the fourth hemibranch (LH4) in comparison with the 1st hemibranch (LH1). * denotes p value < 0.05. Black dots represent each individual sample point, unfilled diamond shape indicates outliers. (D) - PCoA of gill hemibranch bacterial communities separated by individual fish sampled. Shape denotes hemibranch arch location (LH1 -LH4). Ordination demonstrates a high level of homogeneity between bacterial communities of arch samples.

Taxonomic abundance

Overall the ASV's were classified as 9 phyla, 45 orders, 78 families and 163 genera. The two phyla *Proteobacteria* and *Actinobacteria* constituted >80% of the total abundance. Bacterial abundance data indicated that the gill hemibranch communities were largely homogenous across different arches. Increase in the relative abundance of phyla *Proteobacteria* at the 4th hemibranch was noted as the only significant change in taxa at this level (**Figure 2.5A**; Wilcoxon, $p<0.05$). Detail from subsequent mean relative abundance data at the genus level indicates the most prevalent taxa were; *Colwellia* (22.2%), *Cutibacterium* (7.3%), *Arcobacter* (6.3%), *Vibrio* (5.6%), *Aestuariicella* (4.6%), *Pelomonas* (3.3%), *Photobacterium* (3.1%) and *Methyloversatilis* (2.9%), combined

with Unknown taxa (7.1%) and taxa <1% (Other; 37.6%) (**Figure 2.5B**). The only significant changes in the relative abundance between hemibranch arches at the genus level was a marked increase in *Colwellia* at the LH4 hemibranch. The front hemibranch LH1 had a higher abundance of *Cutibacterium* present, as well as a higher composition made up of less abundant bacteria (Other), although not significant. This is likely to be the source of the higher taxa diversity observed in the LH1 and LH2 hemibranch samples in **Figure 2.5B**.

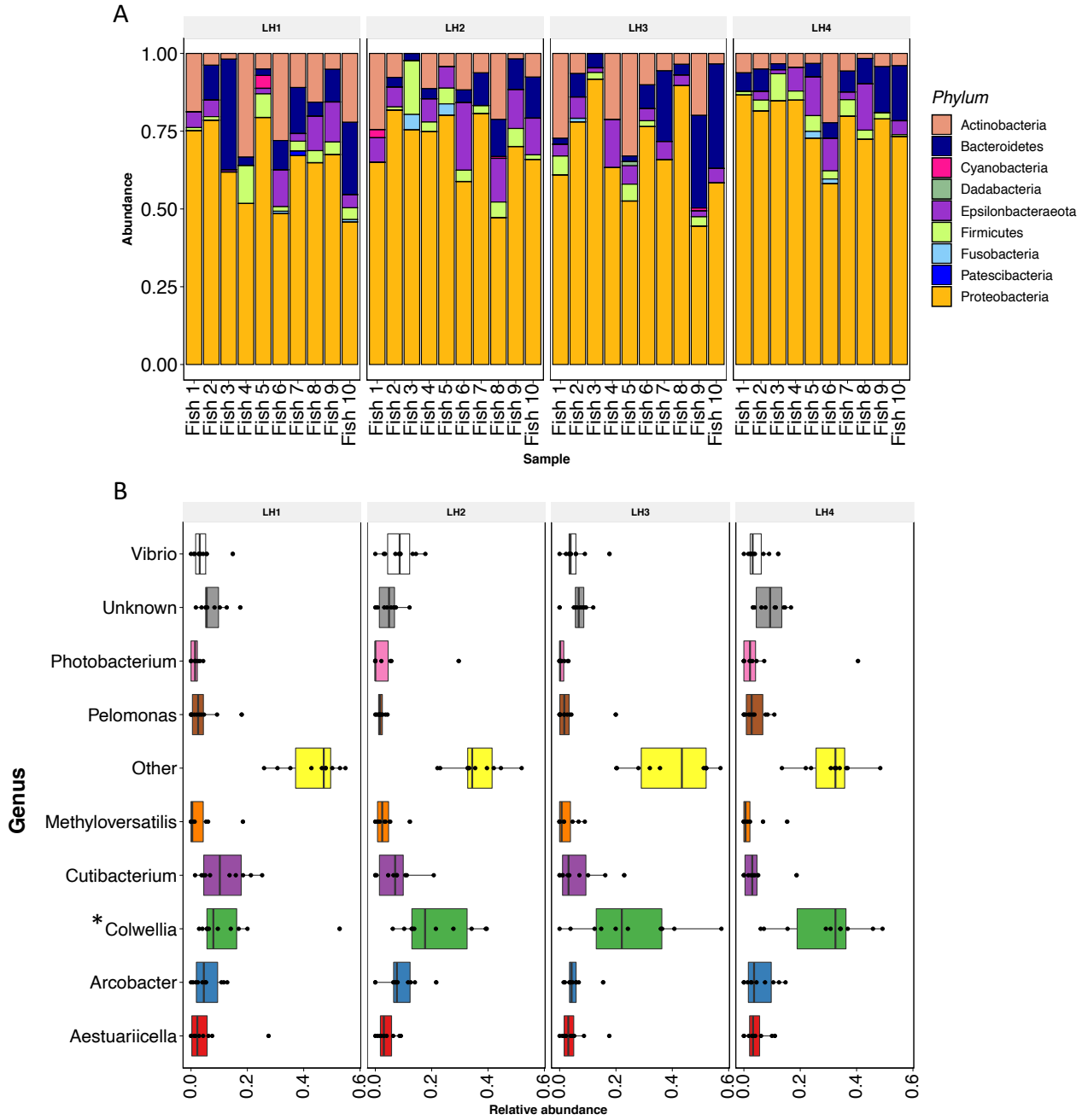

Figure 2.5. (A) Relative abundance of bacteria at phylum, only taxa with > 1% abundance are shown. (B) relative abundance of most prevalent genus level taxa grouped by hemibranch location. Figure is separated into the 1st, 2nd, 3rd and 4th LHS hemibranch for all samples. Black dots represent each individual sample point. * denotes significant taxa between hemibranch arches ($q < 0.05$).

2.5. Discussion

Gill disease is a globally significant issue affecting both marine and freshwater aquaculture species. The role that commensal microbiota plays in gill health, and their association with parasitic, bacterial and viral pathogenesis requires an improved understanding. In the present study we compared the microbial communities residing within and upon the gills. Several commonly employed sampling techniques, both terminal and non-terminal, were compared. Bacterial communities derived from the gill mucus swabs (GMS) were similar in profile to those observed from RNALater preserved tissue (PGF), both in terms of overall alpha diversity and taxa composition. However, bacterial communities derived from the seawater Davidson's fixed tissue (FGF) were significantly less diverse compared to the other sampling methods. Hydrated, formaldehyde-based fixatives such as seawater Davidson's solution are routinely used for cytological preservation for subsequent histological analysis. Fixatives of this nature can hydrate and dissolve much of the mucus overlaying mucosal surfaces (Fernandez et al., 2019a; Lee et al., 1995; Leist et al., 1986; Lumsden et al., 1994). It is likely that gill tissue fixed in seawater Davidson's fixative and those of a similar nature lose much of the resident bacterial community occupying the mucus layer. In contrast, RNAlater preserved tissue appears to retain a greater diversity of bacterial taxa and therefore it is possible that more of the mucus layer and mucus embedded taxa remain ex vivo.

From both practical and diagnostic viewpoints this study suggests that swabbing the mucosal surface of the gill is a suitable collection strategy to provide a representative sampling of the associated microbiome. The community between individual fish was shown to be more variable than the distinction between a tissue vs swab sample taken from the same individual. The microbial community of the nasal mucosa in humans collected via mucosal swabbing had a high level of agreement to samples collected via tissue biopsy (Bassiouni et al. 2015). In an attempt to standardise sampling effort when collecting skin microbiome samples, Ogai et al. (2018) demonstrated that an adhesive tape stripping method was directly comparable to collection via skin swabbing. These studies in combination with the current study shared a similarly low relative sample size as a limitation, but further support our results that a swab-based methodology can be employed in place of wound generating biopsies or terminal necropsy to attain a representative microbial profile from the gills. Swab collection as a non-terminal technique enables sampling the same individuals repeatedly for longitudinal studies. Consideration should be afforded to any potential for damage of the branchial mucosa that may affect microbial profiles in such an instance.

This study suggested that mucus swabbed from the gills contains a bacterial profile similar to that found upon whole gill tissues. Mucus swabs were therefore used to compare bacterial communities across different arches within individual fish to further refine a future sampling approach for gill bacteriomic studies. Swabbing of gills in situ or in vivo typically employs collection of material from the mucosal filamental/lamellar surface of single gill holobranchs or hemibranchs. The requisite assumption being that the collected material will be representative of the collective respiratory surface from all arches in each fish. The potential for differences between biological and environmental profiles for individual arches within entire gill assemblages is largely unknown. Teleost gill circulation for example may not in fact be homogenous across all gill arches (Olson, 1991). Likewise, hydrodynamic resistance and flow fields around the gill arches of teleost fish, such as in tilapia, may also differ depending on cardiovascular demand (Strother, 2013). These factors among others such as water velocity through the filaments of each arch may influence the distribution and diversity of microbial colonisation. Heterogenous parasite distributions across and within gill hemibranchs have been described previously. Forwood et al. (2012) quantified the burden of a monogenean parasite (*Lepidotrema bidyana*) in silver perch (*Bidyanus bidyanus*) noting higher mean parasite abundance on both sides of the first holobranch. Similarly, hyperplastic lesions associated with AGD were more numerous in the dorsal region of the second anterior hemibranch excised from affected Atlantic salmon in situ smolt (Adams and Nowak (2001). Anecdotal observations in vivo suggest that grossly visible gill lesions in AGD affected Atlantic salmon can persist for weeks on the posterior hemibranchs. In naturally affected fish in commercial sea pens, early lesions are seen in dorsal and ventral corners of the second to fourth gill arches, primarily on the posterior hemibranch (R.Taylor, pers. comm.). Results from this study were observed in AGD affected salmon, and therefore may not encompass all experimental scenarios relating to gill microbiota profiling, with further verification potentially required.

Our study demonstrated that the resident microbiota colonising the first anterior hemibranch of Atlantic salmon smolt had significantly higher taxa richness and diversity than in the farthest posterior hemibranch arch (LH4). To the best of our knowledge this variation in bacterial richness and diversity across the gill basket has not been previously described. It is possible that water flow across the frontal hemibranchs may be higher volume than that of posterior arches (Paling, 1968) and therefore a higher exposure to typically highly diverse waterborne bacterial species (Wilkes Walburn et al., 2019) during gill ventilation. The taxa diversity of the hemibranch arches decreased toward the rear of the gill basket in this study. Rear hemibranch arches may not have exposure to as many new or transient bacterial agents potentially coming from the milieu,

or that the functional proportion of these areas are subject to reduced perfusion activity as noted in Nilsson (2007), thus impacting bacterial activity to such regions. *Colwellia* sp., a marine psychrophilic bacteria previously associated with coldwater finfish (Bowman et al., 1997; Minniti et al., 2017), was highly abundant in posterior arches. This genus may be occupying a commensal niche in this environment, although this requires further investigation. The exact nature of this finding requires further elucidation of the physical and chemical pathways and functions which take place between these arch locations. Study specific consideration should be made into the suitability of using one singular arch as a microbial representative fish in vivo sampling designs, as our results demonstrate that there is potential for differentiation in bacterial community profiles between different arches. Further work may be warranted to investigate the effect that the environmental conditions or system play, as these samples were derived from seawater adapted smolt in a flow through experimental facility.

This study suggests that a representative bacterial community (in abundance and diversity) can be profiled from gill mucus when collected using a swab. This may provide greater flexibility for experimental design, easier sample collection and a welfare friendly approach to microbiomic gill studies. Gill mucus is largely lost from samples fixed in Davidson's solution limiting its suitability as a retention method for bacterial communities inhabiting gill mucus. Bacterial community profiles in the posterior arches trended toward less diversity and richness compared to the anterior communities. Taken with previous studies, these results suggest that the posterior arch extremities (LH4 hemibranch arch) should be avoided as a stand-alone sampling location when considering the branchial bacterial community in its entirety.

2.6. Acknowledgements

We gratefully thank Tassal Group Ltd for providing funding for this study and the Institute for Marine and Antarctic Studies (University of Tasmania), for the provision of a PhD scholarship. The authors wish to thank Tansyn Noble for review of this manuscript, and the CSIRO salmon health facility technical staff (Chris Stratford and Lambertus Koster) for their contribution to husbandry of salmon stocks used in this study.

Chapter 3. Profiling the branchial bacterial communities of Atlantic salmon (*Salmo salar* L.) following administration with antimicrobial agents

3.1. Abstract

Microbial gill diseases caused by either opportunistic or specific pathogens are an emerging area of concern for aquaculture producers in part due to their sometimes complex and/or cryptic nature. Many antimicrobial treatments used in aquacultural settings are broad spectrum in nature. The effect of such therapeutics upon reduction and recolonisation of commensal or pathogenic microbiota post-treatment has received little attention to date. Commensal bacteria are an integral component of the barrier function of mucosal surfaces in animals. This study evaluated the effect of several commercially relevant antimicrobial treatments upon the diversity and composition of branchial bacteria of Atlantic salmon. Here we exposed Atlantic salmon smolt to a number of commercially relevant antimicrobial treatments including chemotherapeutants (chloramine-t and hydrogen peroxide) and antibiotics (oxytetracycline and florfenicol) *in vivo*. Subsequently we examined the change in bacterial load, 16S rRNA gene abundance, and taxonomic diversity post-treatment upon the gills. Results revealed a decrease in culturable bacterial colonies after antimicrobial treatment, and a downstream decrease in bacterial richness and abundance post-treatment, with colonisation of several prominent pathogenic taxa including *Vibrio* and *Tenacibaculum*. Temporal tracing over a 14-day period demonstrated that the bacteriome of gill mucus is sensitive to change, and altered by antimicrobial treatment and handling. This study identified candidate antimicrobial treatments which could be implemented in future studies to illustrate the effect of dysbiosis on microbial gill diseases.

3.2. Introduction

Global finfish aquaculture continues to increase rapidly to meet market demands and the need for a sustainable, high yield protein source for a burgeoning global population. Production stressors, life cycle stages, adverse water quality, diet, and disease are some factors affecting the overall health of intensive aquaculture systems (Beck and Peatman, 2015). Compromise and infection on the gill can lead to reduced productivity and economic losses (Rozas-Serri, 2019). The increasing use of high-density animal production systems producing high levels of waste effluent can lead to pathogen proliferation and impinge on production success if not correctly processed or recycled (de Bruijn et al., 2018; Noga, 2010). Pathogen infiltration of fish often occurs through mucosal barriers, including the gill, skin and gastrointestinal tract (Merrifield and Rodiles, 2015). Fish gill surfaces are in constant contact with the aquatic external environment containing an abundance of microbes including pathogenic agents. Mucosal gill surfaces can be a portal for pathogens to colonise and infiltrate leading to localised or systemic disease, and compromise to the gill can impact upon physiological processes due to the multifunctional nature of the organ. Diseases and disorders of the gill are therefore often multifactorial and complex in nature (Mitchell and Rodger, 2011). Some gill conditions such as complex gill disease (CGD) have multiple known aetiological agents (e.g. *Neoparamoeba perurans*, Candidatus *Piscichlamydia salmonis*, *Desmozoon lepeophtherii*, salmon gill poxvirus and Candidatus *Branchiomonas cysticola*) (Gjessing et al., 2019) which interact in a co-infection. Other presumed single-agent gill conditions such as columnaris disease (*Flavobacterium columnare*) and yellow mouth (*Tenacibaculum maritimum*) have strong environmental influences, including temperature and salinity, which can affect infection severity (Bandilla et al., 2006; Wynne et al., 2020b).

The concept of an innate immune benefit in-part provisioned by commensal bacteria within the mucus layer is known colloquially as barrier health (Beck and Peatman, 2015). The commensal bacterial community inhabiting these areas represent a component of the defence barrier against pathogens (Cabillon and Lazado, 2019), and are thought to be most effective when the microbial community is highly diverse (Merrifield and Rodiles, 2015; Wilson and Laurent, 2002). These microbes colonise gill mucus and provide competitive exclusion toward opportunistic pathogens, synthesis of antimicrobial compounds (e.g. bacteriocins, antimicrobial peptides, hydrogen peroxide) and assist with immune functions such as phagocytic activity (Cabillon and Lazado, 2019; Gómez and Balcázar, 2008). The diversity of the commensal microbiota can be profiled using next generation sequencing techniques providing an indicator of health for a given aquaculture species (Derome et al., 2016).

Disease can occur within intensive aquaculture systems, and treatment of animals in both a prophylactic and therapeutic nature is essential to maintain welfare and promote optimal growth. Treatment options include the use of chemotherapeutics (e.g. oxidative compounds), antibiotics as well as manipulation of water quality parameters (i.e. transfer to salt/freshwater, change in temperature). Chemotherapeutic treatment is well documented for a range of prominent bacterial and fungal infections, an example of this is the use of chloramine-trihydrate (Cl-T) as an immersion bath treatment for salmonid diseases such as Bacterial Gill Disease and Columnaris Disease (caused by *Flavobacterium* spp.) (Bowker et al., 2008; Bullock et al., 1991; Genaro Sanchez et al., 1996). The intermediary breakdown of chloramine-t (to paratoluenesulphonamide and hyperchlorite ions) results in an expectorant effect, promoting gill mucus production (flushing) and a bactericidal action. Ectoparasites are also commonly treated in this fashion. Hydrogen peroxide (H_2O_2) is an chemical which releases oxygen free radicals, causing a direct oxidative effect on microorganisms, and breaking down into environmentally friendly by-products (H_2O and O_2). It has been used within the salmonid industry for decades as one of the most reliable treatments of sea lice (*Lepeoptheirus salmonis* and *Caligus* spp.), and to a lesser extent amoebic gill disease (AGD) (Adams et al., 2012; Kiemer and Black, 1997; Powell et al., 2015). Antibiotic treatments are often utilised to treat more systemic or internal bacterial infection events, which can be administered via immersion bath or oral feed delivery. Commonly used compounds include florfenicol and oxytetracycline, which have a bacteriostatic killing action used to effectively treat conditions such as furunculosis (*Aeromonas salmonicida*), vibriosis (*Vibrio* spp.), piscirickettsiosis (*Piscirickettsia salmonis*) (Henríquez et al., 2016; Lundén et al., 1999; Noga, 2010; Nordmo et al., 1994; Schmidt et al., 2017). However, due to the broad-spectrum killing action of many antimicrobial agents, non-target taxa can be affected by the treatment process (Noga, 2010). This may inadvertently lead to a microbial imbalance, often termed as 'dysbiosis' (Egan and Gardiner, 2016; Francino, 2016), which can lead to further health issues and susceptibility to infection. Therapeutic Cl-T treatment in rainbow trout (*Oncorhynchus mykiss*) left the skin in an infection prone state, and was colonised by secondary opportunist agents including *Tenacibaculum* and *Pseudomonas* (Genaro Sanchez et al., 1996). Mohammed and Arias (2015) demonstrated that microbial dysbiosis of channel catfish *Ictalurus punctatus* induced by antibacterial bath immersion was associated with increased susceptibility to experimentally induced columnaris disease. Similarly, Schmidt et al. (2017) demonstrated that after challenging black molly (*Poecilia sphenops*) with *Vibrio anguillarum,* treatment with streptomycin led to significant mortality. Survival in the antibiotic treated fish which were also provided a probiotic additive treatment showed a much higher survival rate, indicating that the subtle microbiome supplementation lessened the impact

of microbial dysbiosis on the fish. Despite these examples, there is a limited understanding of the effect broad spectrum antimicrobial compounds have in the context of bacterial dysbioses upon mucosal surfaces, especially those of the gills. Furthermore, the temporal response of gill microbiota post-treatment is largely undescribed past the initial treatment window, along with the impact of topical vs systemic antimicrobial treatments, and sources of community recolonisation.

In this study we used a combination of microbiological, molecular and amplicon sequencing techniques to determine the impact of several antimicrobial treatment regimes. The major aims of the study were to determine if broad spectrum antimicrobial compounds affect the commensal gill community, and to evaluate the post-treatment response of the bacterial community following such treatments. This research will have implications for future studies investigating the intricacies of microbial gill disease, and offer a model process to induce microbial dysbioses in an applied scenario. It will also provide insights regarding the significance of commensal bacteria as a component of the mucosal health barrier.

3.3. Materials and methods

All activities relating to fish maintenance and sampling in this trial were approved under the CSIRO QLD Animal Ethics Committee, permit number #2018-18.

3.3.1. Fish source

A cohort of 200 Atlantic salmon (*Salmo salar*) parr were on-reared at the Bribie Island Research Centre in purpose-built recirculation systems for a period of approximately 9 months. Fish were fed daily to satiation on a commercial pelleted feed and prepared for smoltification after attaining a mean weight of 150 g. Fish were exposed to a constant photoperiod (24^{L}: 0^{D}) at an intensity of 3300 lumen for a period of 5 weeks and then transferred from the freshwater RAS to marine flowthrough water via constant system water exchange within the 5000 L tank. Post smolt were then allowed to acclimate to marine water over a period of two weeks prior to trial commencement, with water temperature held at 15°C ±1, dissolved oxygen 90-110% sat, TA-N <0.50 ppm, and salinity 35-36 ppt.

3.3.2. Trial stocking

Upon trial commencement 147 Atlantic salmon post-smolt (229.4±0.4 g) were anaesthetised using 17 $mg \cdot L^{-1}$ AQUI-S® (Aqui-S Ltd., Lower Hutt, NZ, New Zealand), individually weighed and stocked into an array of seven identical 500 L tanks (n=21) which were provided with identical flowthrough seawater and had independent drainage systems. During the trial, the water temperature was maintained at a range of 15±0.5°C and the dissolved oxygen 90-110% sat by chilling infrastructure and oxygen monitoring probes with automated oxygen release. The system

was operated as flow-through, with seawater pumped from approximately 300 m off the beach adjacent to the research station then through a series of 16 spin disk filters (40 μm) and 10 multimedia filters (~10-15 μm), after which received ozone treatment from two 100 gO_3.h generator units (Wedeco OCS-GSO30). The ozone treated seawater was then pumped via ultra violet disinfection units, providing 80 $mJ.cm^2$ dosed to two (~8 m^3) granular activated carbon vessels for a contact time of >9 mins to remove unwanted by-products from the ozone treatment. Finally, the seawater was pumped to a header tank, which fed directly into a pipe system delivering treated seawater to this experiment. The array was light-controlled and maintained a 14^L:10^D photoperiod throughout.

3.3.3. Antimicrobial treatments

Antimicrobial treatments in the form of in-feed antibiotics and immersion therapeutic baths were conducted to reduce branchial bacteria loads. Antibiotic coated feeds were prepared for this work by adding pelleted feed (3 mm Spectra, Skretting P/L, Cambridge, TAS) into a Hobart mixer bowl (Hobart, Ohio, USA) with the required addition of pre-warmed fish oil (60°C) combined with an emulsion of concentration of either 79 $ppm.kg^{-1}$ oxytetracycline hydrochloride (OTC) (CCD, NSW, Australia) or 10 $ppm.kg^{-1}$ of florfenicol (FF) (Abbey Labs, NSW, Australia) respectively. Each compound emulsion was then poured into separate pellet bowls, where a sealed lid was added and the chamber was evacuated of air using a vacuum pump at 350 P.S.I for 5 minutes, until visible air escaping the pellets was no longer observed. Antibiotic coated pellets were stored in the dark at -20°C, and the daily ration was taken from the freezer to be loaded into the autofeeder hopper. Antibiotics pellets were offered daily to fish in OTC and FF tanks, completing a ten-day course duration.

At the completion of the antibiotic course, the therapeutic chemical bath treatments were carried out to synchronise sample timing (**Figure 3.1**). The three oxidative immersion bath treatments used were as follows; chloramine-trihydrate (Cl-T, Sigma Aldrich, USA) in saltwater where the 25 $mg \cdot L^{-1}$ dose was verified by measuring (total chlorine – free chlorine) x 3.97 (Y.S.I 9500 photometer) for 60 min, hydrogen peroxide (H_2O_2, Solvay Interox, Australia) divided into both a saltwater treatment at 1250 ppm for 15 min, and a freshwater treatment at 500 ppm for 20 min. Fish from each stocked tank (n=21) were transferred to four identical static baths made up the three aforementioned bath treatment concentrations, along with a sham bath (bath control) containing only filtered saltwater for 60 mins. Fish behaviour was monitored closely throughout the duration of the bath treatments, and upon completion, 18 fish were netted back into their holding tank and three fish were sampled from each treatment. After initial treatment fish from all groups were held in their respective experimental tanks for the duration of the trial. All tanks

including antibiotic feed treatments were offered a daily ration of 1% bodyweight (BW) (3 mm Skretting Spectra) via auto feeder system (Arvotec wolf controller, Arvotec-Oy). Daily maintenance included recording water quality (temperature and dissolved oxygen), observing fish for irregular behaviour, cleaning tank systems and collection of any uneaten feed at the conclusion of the autofeeder activity period. This collected feed was retained into a mesh sieve (1 mm aperture), where it could be transferred to individual trays and dried overnight at 105°C to obtain dry weight and allow total uneaten feed to be calculated.

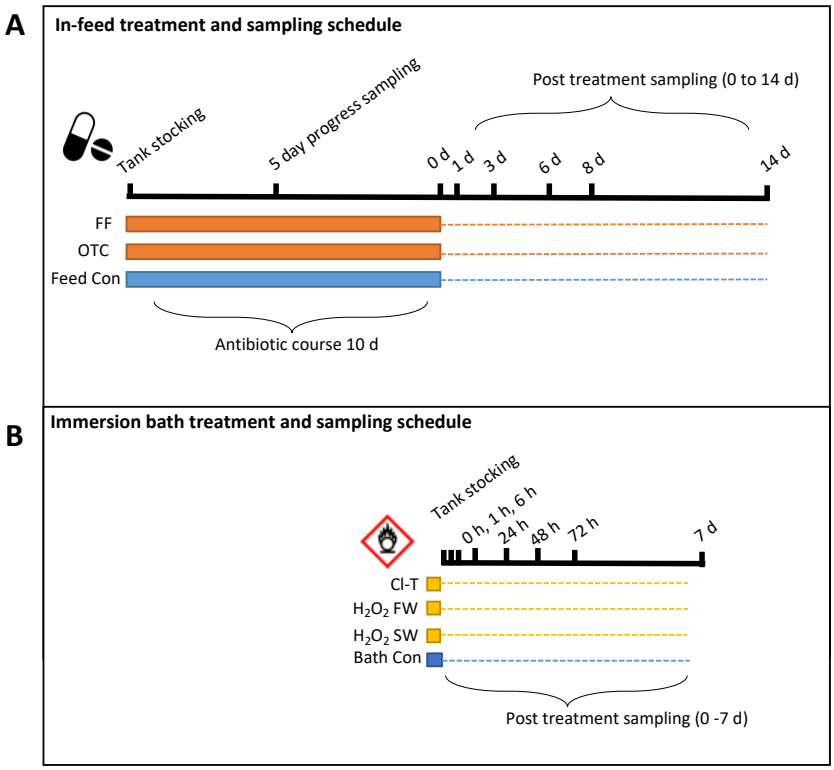

Figure 3.1. Schematic illustration of experimental sampling regime for each group of (A) infeed antibiotics and (B) oxidative bath treatments. Solid banding indicates the disinfection 'period' for each group, while the dotted line represents the longitudinal trial sampling period to its completion. Black ticked lines and respective times indicate the sampling timepoint when fish were sampled.

3.3.4. Sample collection and preparation.

To account for the systemic nature of in-feed antibiotic administration, the sampling schedule for feed treatments was extended to a 14-day timeframe, whilst oxidative bath treatments were expected to have shorter term impact and recovery and thus sampling was over a shorter duration (7 days) targeting the immediate post-bath period.

3.3.5. Aerobic count plates

Gill mucus was sampled by taking a swab of the first right hand side (R1) hemibranch (three rotations along the length of the arch) and placing the swab into 1 ml of filtered, autoclaved

seawater in a 1.5 ml tube. Each tube was then vortexed for 15 seconds, before a 500 µl aliquot was pipetted onto an aerobic count film (3M petri-film®) and incubated at 35±0.1°C for 48 h before colonies were visually counted in and recorded (calculated as $CFU.mL^{-1}$)

3.3.6. Gill mucosa sampling (for 16S bacterial community and real time PCR)

On each nominated sampling timepoint (**Figure 3.1**), three individual fish from each group were humanely killed (by immersion in 100 ppm AQUI-S™) and then sampled. A mucosal gill sample was taken by swabbing all anterior and posterior hemibranch from the entire right-hand side of the gill basket (8 surfaces). This was achieved using a sterile cotton swab (Westlabs), where the swab was rotated three times on each of the 8 hemibranch surfaces. Swabs were then transferred to a 1.5 ml screw cap tube containing 1 ml of RNAlater solution, and stored at 4°C for 24 hours before being frozen and stored at -80°C until further processing could occur.

Tank water samples were collected by filling 3 sterile HDPE collection bottles with 500 ml volume from each individual tank, and passaging the contents of each bottle through a 0.22 µm Sterivex™ (Millipore) filter membrane unit using a peristaltic pump (RP-100 series, Lachat Instruments) to retain bacterial cells. The Sterivex™ filter chamber was then flooded with 2 mL of RNAlater solution and then stored at −20 °C prior to DNA extraction.

3.3.7. DNA Isolation

Bacterial DNA was extracted from both mucosal cotton swab samples as well as from 0.22 µm sterivex water filter units. Mucosal swab samples were extracted using the Qiagen DNeasy spin column extraction kit, using a modified protocol to the standard blood and tissue documentation. Briefly, swab samples were agitated and centrifuged, with excess RNAlater fixative pipetted off to waste. The process then followed the blood and tissue documentation until completion. Sterivex filter samples were extracted using the Qiagen Sterivex DNA extraction kit, as per manufacturers protocols. Genomic DNA quality and concentration was verified using a nanodrop ND1000 spectrophotometer (Life Technologies).

3.3.8. SYBR green qPCR assay for 16S rRNA and ELF housekeeping gene

Target gene for the assay was the V3-V4 hypervariable region of the 16S rRNA bacterial gene, defined by a 174 bp fragment using the following primers 341f 5'-CCTACGGGAGGCAGCAG-3' and 515r 5'-ATTCCGCGGCTGGCA-3' as described in (López-Gutiérrez et al., 2004). The Atlantic salmon elongation factor gene EL1-a (ELF) described in Bland et al., (2012) was used as the reference gene in this assay, amplifying a 66 bp fragment using the primer set S-ELF.f 5'-GGCCAGATCTCCCAGGGCTAT-3' and S-ELF.r 5'-TGAACTTGCAGGCGATGTGA-3'.

Extracted DNA was diluted to a working concentration of 10 ng.ml for all samples. Real time PCRs were carried out in a ViiA™ 7 Real-Time PCR Machine (Applied Biosystems). qPCR was performed in a single-plex 25ul reaction containing 1.25 ml of 10 uM forward and reverse primer, 8 ml of RNase-free H_2O, and 12.5 ml of SYBR Green qPCR Mastermix including hotstart Taq polymerase (Bioline). Each reaction contained 2 ml of normalised template DNA (30 $ng.ul^{-1}$). PCR reactions were subjected to the following thermal cycling: 95°C for 10 min, then 35 cycles of 95°C for 30 s, 72°C for 40 s and 76°C for 35 s. A melt curve was also included in the assay, set at 95°C for 15 s, 60°C for 1 min and 95°C for 1 s. PCR reaction volumes were loaded into a 384 well plate in triplicate for both the target and reference gene on each biological sample.

3.3.9. PCR preparation and 16S rRNA amplicon sequencing

After gDNA was extracted, it was amplified by PCR using Illumina fused primers which targeted the V1-V3 hypervariable region of the 16S rRNA gene. The sequences for the forward and reverse primers were as described in Table 3.1.

Table 3.1. Primer sequences used in the current study for PCR submission amplifying the V1-V3 region of the 16S rRNA gene

Forward primer 5'-3' ("27F-adapt")		
Illumina forward overhang adapter	27F	Refs
TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG	AGAGTTTGATYMTGGCTCAG	(Lane et al., 1985; Zheng et al., 2015)
Reverse primer 5'-3' ("519R-adapt")		
Illumina reverse overhang adapter	519R	Refs
GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG	GWATTACCGCGGCKGCTG	(Lane, 1991; O'Farrell et al., 2019)

DNA concentration of each sample was quantified using a nanodrop spectrophotometer (ND-1000) and were diluted to 10 $ng.\mu l^{-1}$ as template for the PCR reactions, using Platinum Taq Hi fidelity mastermix (Thermo Fisher). Cycling was completed as per the following conditions; 94 °C for 90s; 25 cycles of 94 °C for 30s, 52 °C for 30s, 72 °C for 90s; and a final extension of 72 °C for 10 min. Sequencing was carried out on an Illumina Miseq platform at the Ramaciotti Center for Genomics (UNSW, Sydney), generating forward and reverse reads of 300 bp in length.

A negative control of ultrapure analytical grade water was included within the PCR reaction. After cycling was completed, PCR product amplification was verified via gel electrophoresis for the target amplicon. A negative process control (unused cotton swab opened and placed into a tube with RNAlater) and a mock community standard (ZymoBIOMICS Microbial Community

Standard, Zymo Research) containing a known composition of 8 bacterial species was sequenced to validate sequencing effort and quality.

3.3.10. Bioinformatics pipeline

Raw Illumina amplicon sequencing data files were processed using the open-source software pipeline "Quantitative Insights into Microbial Ecology 2" QIIME2 (Caporaso et al., 2010). Paired end sequences from the forward and reverse reads were merged for each sample and were denoised using the q2-dada2 plugin (Callahan et al., 2016) with default parameters. Quality control including chimeric sequence removal from the dataset was completed during dada2 processing, along with subsequent removal of host DNA and exclusion of chloroplast and mitochondrial sequences. Amplicon Sequence Variants (ASV's) were classified taxonomically using the classify-sklearn method in the QIIME2 q2-feature-classifier plugin using default parameters (Bokulich et al., 2018). The SILVA 16S rRNA 99% taxonomy database release 132, (Quast et al., 2012), was used as reference sequences for taxonomic classification.

3.3.11. Statistical analysis

All statistics were performed in R version 3.6.0 (R Core Team, 2019). Daily feed consumption was calculated as % bodyweight consumed per day by subtracting collected, dried uneaten pellets from the total ration fed to each tank. The mean ±SD of this metric was compared between treatments using a one-way ANOVA and Tukey post-hoc testing. Bacterial count plate data was arcsine-transformed and the $CFU.mL^{-1}$ values analysed using a one-way ANOVA with Tukey PSD post hoc testing. Real time qPCR data were analysed as log-fold change between treatment groups of the ratio of the gene of interest (16S rRNA) after against the housekeeping (salmon Ef1α) control, and assessed using two-way ANOVA with treatment and timepoint as factors ($padj < 0.05$). Samples from the 16S NGS data were rarefied using R package QsRutils (Quensen, 2020) performed on a maximum subsampling depth of 7171 sequences per sample (**Figure S3.1**). Obvious contaminant artefact present in the negative control sequences was identified and subsetted from biological samples via the Decontam package (Davis et al., 2018). Using the Phyloseq package (McMurdie and Holmes, 2013) taxonomic assignments were generated and alpha diversity indices calculated (Observed ASV's, Shannon diversity, Faith's phylogenetic distance). The alpha diversity metrics were analysed via non-parametric means (Kruskal-Wallis test) and further pairwise comparisons using a Wilcoxon Test (Rank Sum Test). Beta-diversity comparisons were made via NMDS using Bray Curtis pairwise distances. Differences between groups was analysed using PERMANOVA via the pairwise Adonis package (Martinez Arbizu, 2019). Relative taxonomic abundance was analysed using the DeSeq2 package (Love et al., 2014) to test for differentially abundance bacterial taxa between groups. The origin of gill mucus

samples was investigated by comparing the core branchial bacterial community against the source tank water using the FEAST package (Shenhav et al., 2019). All figures were produced using the R package ggplot2 (Wickham, 2016).

3.4. Results

Feed intake data

Several known antimicrobial treatments administered were in line with the Food and Drug Administration (FDA) recommended delivery (bath or in-feed) and dosage. Feed intakes for antibiotic treatment were examined daily to ensure that fish consumed enough feed to receive the correct dosage listed in Table 3.2.

Mean feed consumption across all tanks remained at or above 1% bodyweight per day for the duration of the antibiotic course (or habituation period for other groups). Mass specific intake (% BW.day) was significantly lower for the florfenicol coated diet comparative to the fish-oil coated commercial pellets (ANOVA, $F_{6,63} = 4.788$, $p<0.001$). There was no difference in intake values between the two antibiotic coated diets (OTC and FF), or between commercial pellet and fish-oil coated commercial pellets.

Table 3.2. Feed intake measured as percent bodyweight per day consumption over the total antibiotic course timeframe. Values represent mean ± SD, where means followed by the same superscript letter do not differ significantly at $p<0.05$ (Tukey-HSD).

Chemical name	Treatment code	%BW.day ± SD	In-feed dose	Comment
Chloramine Trihydrate (seawater bath)	Cl-T	1.16 ± 0.02 ^{a}	n/a	Not coated
Hydrogen Peroxide (freshwater bath)	H_2O_2 FW	1.17 ± 0.01 ^{a}	n/a	Not coated
Hydrogen Peroxide (seawater bath)	H_2O_2 SW	1.15 ± 0.03 ^{a}	n/a	Not coated
Bath control (seawater bath)	B.con	1.13 ± 0.06 ^{a}	n/a	Not coated
Oxytetracycline Hydrochloride	OTC	1.06 ± 0.05 ^{ab}	79 mg.kg^{-1}	Fish-oil coated
Florfenicol	FF	1.00 ± 0.12 ^{b}	10 mg.kg^{-1}	Fish-oil coated
Feed control	F.con	1.13 ± 0.05 ^{a}	none	Fish-oil coated

Bacterial count data and real-time qPCR assay

Total bacterial counts (CFU.mL^{-1}) from the R1 hemibranch surface varied within the trial and across sampling dates, with the time by treatment interaction significant (ANOVA, $F_{32,96} = 2.135$, $p<0.001$). This was characterised by a decline in viable count plate colonies directly after all antimicrobial (bath and in-feed) treatments. Immersion bath treatments including Cl-T, H_2O_2 FW and H_2O_2 SW recorded lowest count numbers directly post-bath (0 hr) which largely

increased over the 7-day trial period (**Figure 3.2A**). The seawater bath control also showed an initial decrease in CFU.mL^{-1} at the start of the trial, increasing significantly at the 1 hr timepoint (p<0.05) followed by relative stabilisation toward the end of the trial period. Antibiotic treatments (**Figure 3.2B**; OTC, FF) were observed at significantly lower levels to the control at the mid-course(p<0.01), completion of the course (0 hr; p <0.001), and 1-day post treatment(p<0.05). All three groups remained relatively consistent between subsequent timepoints, with the feed control group being significantly higher at 14 days(p<0.01). Quantitative PCR data were assessed as the log-fold change of the 16S rRNA gene against a reference housekeeping gene (ELF), and compared back to the respective control from the bath and in-feed groups (**Figure 3.2C,D**). Immersion bath treatments were variable throughout the 7-day period. The H_2O_2 FW group demonstrated lower 16S gene abundance of the at 0 hr, 6 hr and 1-day post-bath to the reference control, but this was not statistically significant. At the day 3 timepoint, Cl-T and H_2O_2 SW groups had a higher log-fold abundance, although this was not significant. At the 2-, 3- and 7-day sample points, all three bath treatments expressed positive log-fold increase in 16S gene abundance. OTC treated gill mucus at the mid-course point in **Figure 3.2D** showed a significant decrease in 16S gene abundance ($padj$<0.05). Both OTC and FF treatments were characterised by a decrease in 16S abundance between the mid-course sample to 1-day post-treatment. Log-fold abundance was slightly higher between day 3 and day 8, before values stabilised close to the control reference point for the 14-day timepoint.

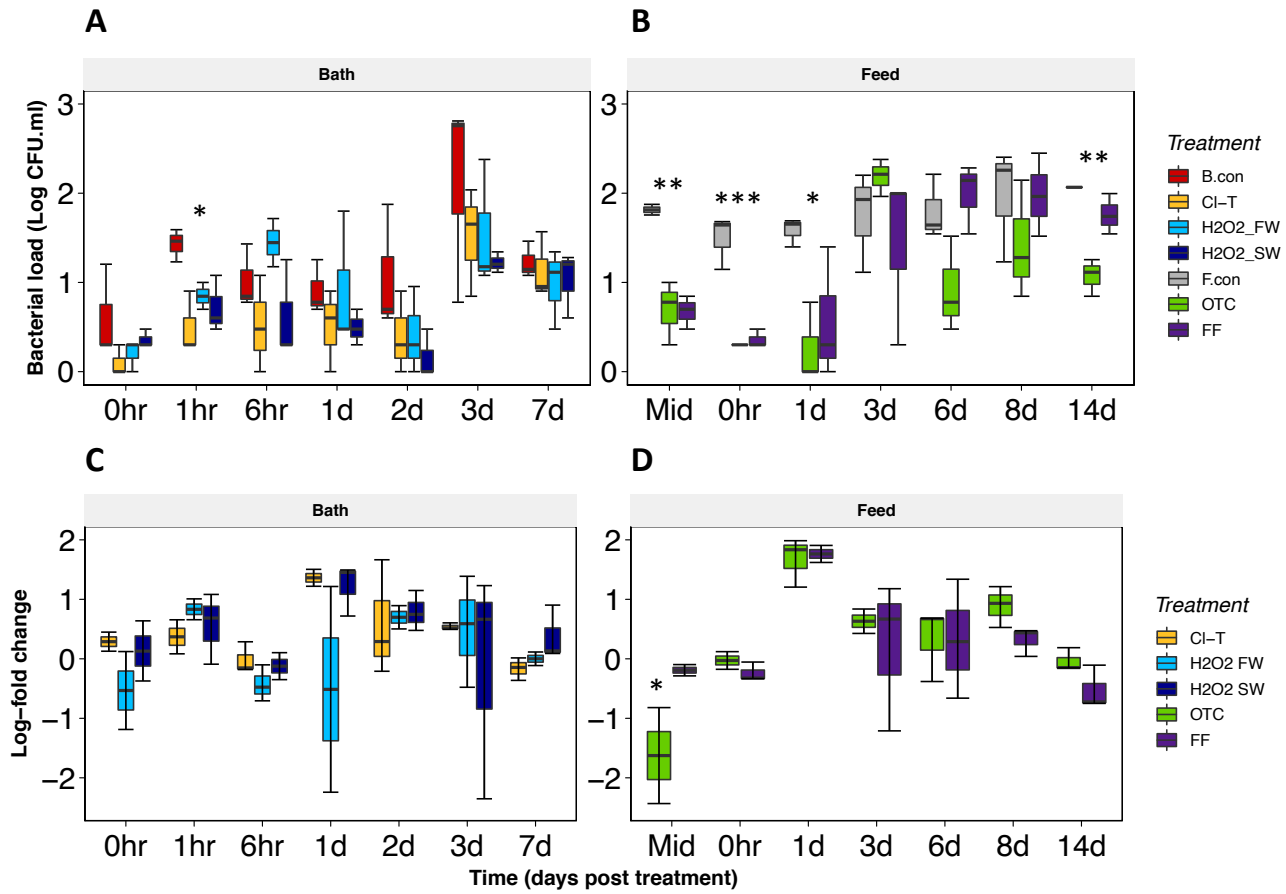

Figure 3.2. Log transformed bacterial count plate data +SE for bath (2A) and in-feed (2B) treatments. Data are presented as estimated marginal means, with the error bars representing the standard error of the mean. At each timepoint a comparison of different treatments (between means of CFU.mL^{-1} counts) were compared and differences are indicated by * ($p \leq 0.05$), **($p < 0.01$), or *** ($p \leq 0.001$). 2C, 2D shows quantitative PCR log-fold 16S rRNA gene abundance of gill mucosa samples from immersion bath (2C) and in-feed treatments (2D) compared to each respective control. An asterisk * indicates a significant difference from the control group at that particular timepoint ($p < 0.05$).

Bacterial diversity of gill mucosa

From the 147 gill swabs and 28 tank water samples we obtained a total of 7,939,968 raw sequence reads. QC and merging sequences resulted in an average of 43,152 reads per sample, with only one sample below 7000 reads. The subsequent ASV table generated 7,296 bacterial taxa from which the diversity and taxonomic analyses were computed.

Alpha diversity metrics were used to assess bacterial richness (Observed ASVs) and diversity (Shannon index) and compare community structure within antibacterial treatments across timepoints (**Figure 3.3**). Timepoints within each group were analysed to determine the magnitude of change post-treatment. Over a 7- day post-bath treatment period, bacterial richness and diversity in fish gills exposed to Cl-T and filtered seawater (bath control) remained static,

with no significant interactions over time (p>0.05). Hydrogen peroxide treated fish in both freshwater and seawater did differ longitudinally in both observed ASVs and Shannon index, and was deemed statistically significant (p<0.05; **Figure 3.3A,C**). Both groups were characterised by an increase in richness and diversity at 1 h and 6 h, before values decreased and remained relatively stable. Within the in-feed groups, ASV richness differed with both antibiotic treatments starting and finishing at similar levels, while the feed control slightly increased over time. Shannon diversity was low in the initial timepoints for all groups, and increased consistently to the 14 day timepoint. Kruskal Wallis testing demonstrated that none of the longitudinal changes in both observed ASVs or Shannon diversity were statistically significant for OTC, FF or the feed control (**Figure 3.3B,D**; p>0.05).

Beta diversity was visualised using ordination of the gill mucus samples via canonical correspondence analysis (CCA) demonstrated that treatments grouped together strongly throughout the experimental period (**Figure 3.3E**). PERMANOVA comparing treatment group and timepoint indicated a significant interaction (p<0.001) for both factors, but the Treatment*Time interaction was not significant (p>0.05). Pairwise adonis for each comparison of gill mucus samples revealed significant differences between H_2O_2 FW and all treatments (p<0.001), as well as H_2O_2 SW and all other treatments (p<0.001). Cl-T was significantly different to the bath control, feed control, FF at OTC treatments (p<0.001). Tank water sample groups (**Figure 3.3F**) were also significantly different from one another when grouped by treatment (PERMANOVA, p<0.001), with the difference being between the Cl-T and OTC tank (p<0.05).

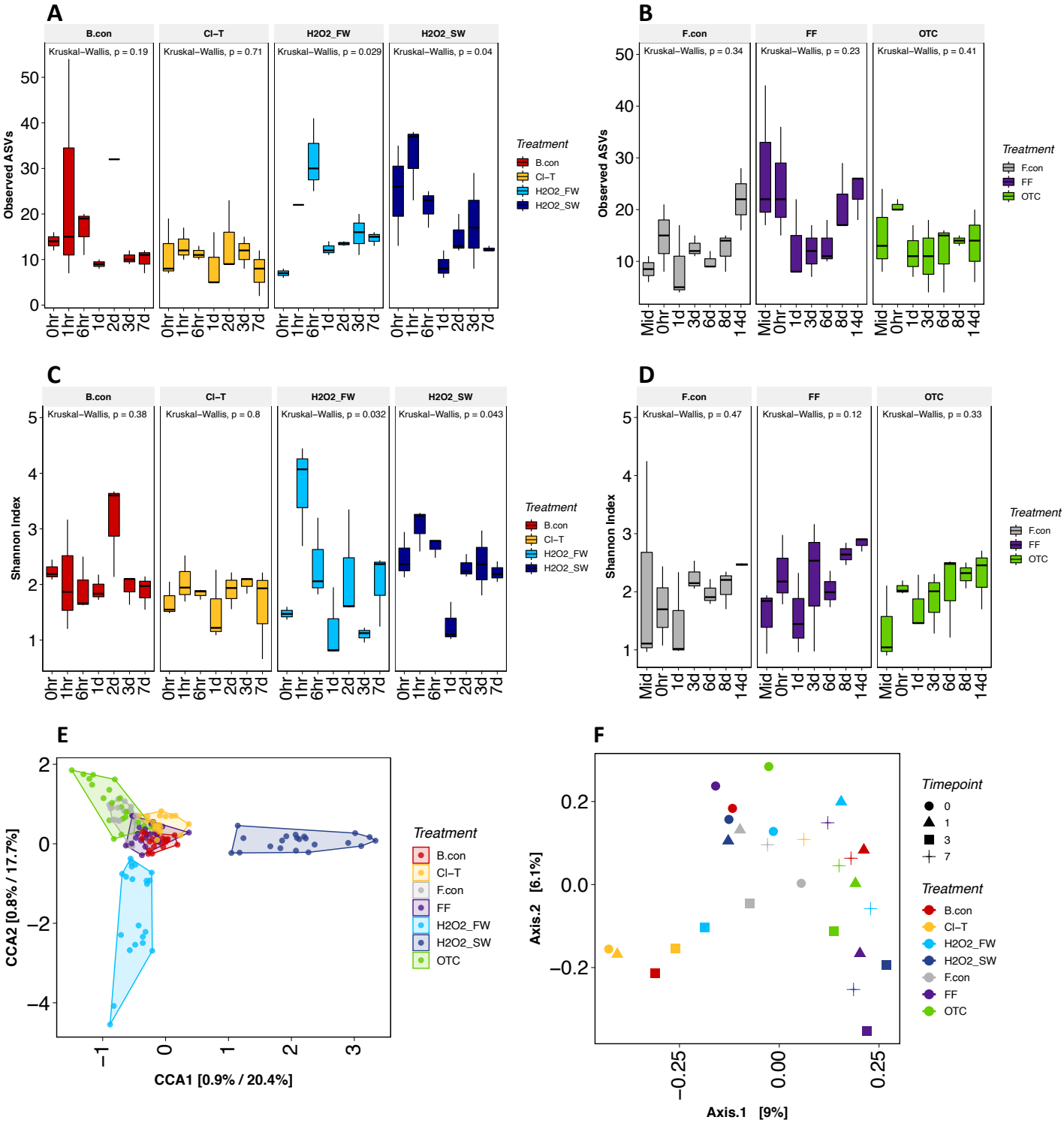

Figure 3.3. Alpha diversity metrics showing ASV richness on gill mucus samples for bath (A) and in-feed (B) antimicrobial treatments, along with Shannon diversity metrics for bath (C) and in-feed (D) groups. Statistical differences were assessed using a Kruskal-Wallis test. Beta diversity metrics via CCA ordination are shown for gill mucus samples (E) and tank water (F) samples

Taxonomic assignment of gill mucus

To further understand differences in bacterial richness and diversity observed in alpha and beta metrics, we examined the relative abundance of bacterial taxa at the phylum level to assess community change within each treatment across the time course. The most abundant phylum associated with gill mucus samples were *Proteobacteria* (37.30%), *Verrucomicrobia* (26.05%), *Actinobacteria* (24.69%), *Bacteriodetes* (9.30%) and *Firmicutes* (2.43%). *Actinobacteria* was present in all timepoints, but in highest abundance in the Cl-T and H_2O_2 SW bath treatments. *Bacteriodetes* and *Firmicutes* were inconsistent in abundance throughout most treatments, with no distinct trends apparent. The phylum *Verrucomicrobia* was also highly abundant, but decreased within the in-feed treatments over the duration of the trial. This taxa decreased in the H_2O_2 SW group post bath treatment, and was absent in the H_2O_2 FW group until 1-day post bath before returning in high abundance. DeSeq2 analysis indicated that both *Verrucomicrobia* and *Proteobacteria* were statistically different between the bath treatment groups (*padj*<0.001). Only the phylum *Verrucomicrobia* was deemed significantly different for in-feed treatment groups (*padj*<0.01). The 100 most prevalent ASVs were classified to genus level and compared, to identify key genera involved in the post perturbation period. Further examination of the 10 most abundant genera revealed that *Rubritalea* were more prevalent at the beginning of the trial for all treatments excluding H_2O_2 FW, and that the abundance of *Pseudoaltermonas* increased markedly at the final sampling points (**Figure S3.2**). When compared to the reference bath control, the dominant genera *Rubritalea* and *Cutibacterium* were differentially expressed in Cl-T, H_2O_2 FW and H_2O_2 SW groups (*padj*<0.001; **Figure 3.4B**). The same taxa were not statistically different for in-feed treatments compared to the feed control (*padj*>0.05). Other prominent taxa included *Pseudoaltermonas*, *Vibrio* and *Tenacibaculum*. These genera were at higher abundance toward the later stages of the sampling period.

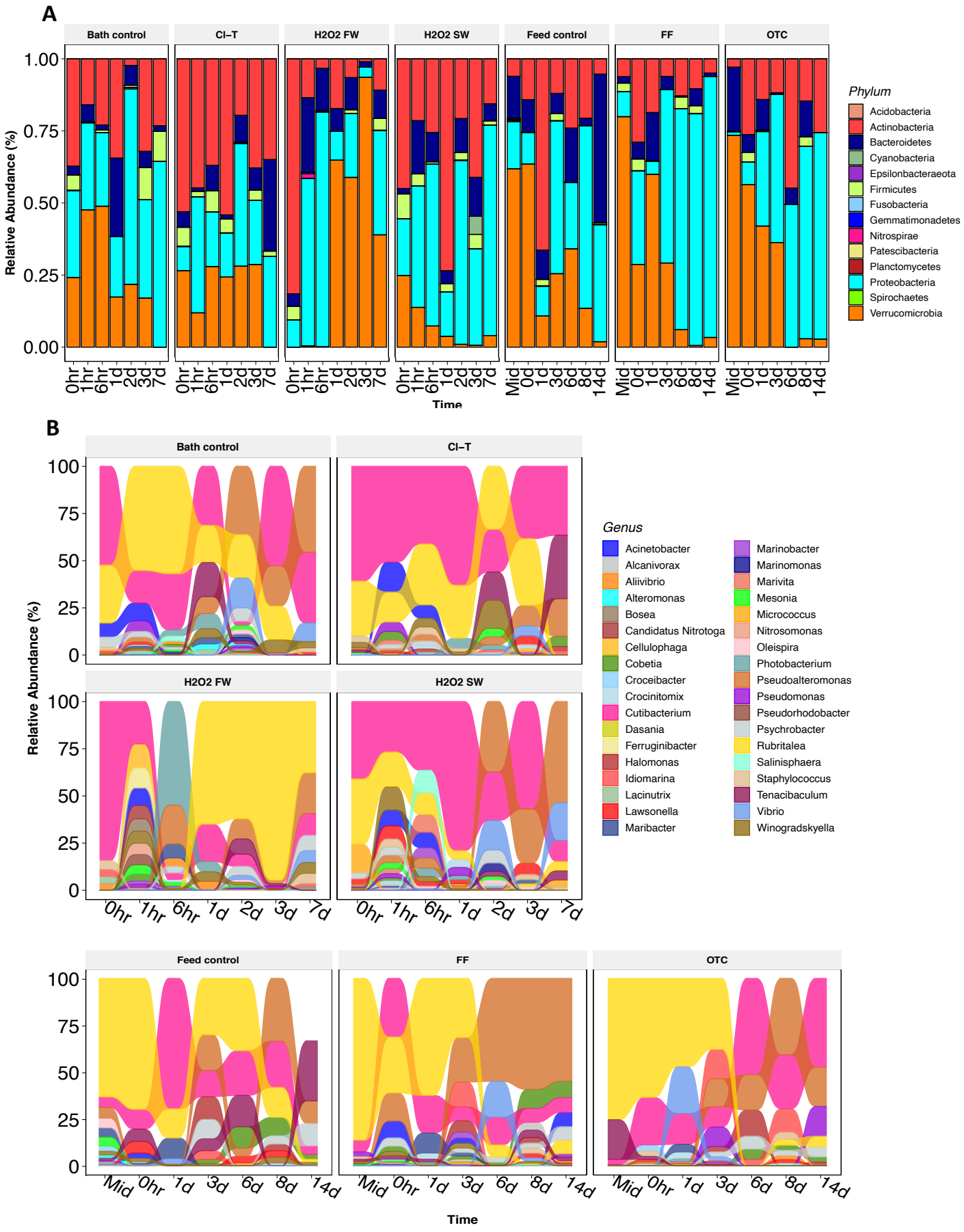
A
Bath control
Cl–T
H2O2 FW
H2O2 SW
Feed control
FF
OTC
Relative Abundance (%)
1.00
0.75
0.50
0.25
0.00
0hr 1hr 6hr 1d 2d 3d 7d
Mid 0d 1d 3d 6d 8d 14d
Time
Phylum
Acidobacteria
Actinobacteria
Bacteroidetes
Cyanobacteria
Epsilonbacteraeota
Firmicutes
Fusobacteria
Gemmatimonadetes
Nitrospirae
Patescibacteria
Planctomycetes
Proteobacteria
Spirochaetes
Verrucomicrobia
B
Bath control
Cl–T
H2O2 FW
H2O2 SW
Feed control
FF
OTC
Relative Abundance (%)
100
75
50
25
0
0hr 1hr 6hr 1d 2d 3d 7d
Mid 0hr 1d 3d 6d 8d 14d
Time
Genus
Acinetobacter
Alcanivorax
Aliivibrio
Alteromonas
Bosea
Candidatus Nitrotoga
Cellulophaga
Cobetia
Croceibacter
Crocinitomix
Cutibacterium
Dasania
Ferruginibacter
Halomonas
Idiomarina
Lacinutrix
Lawsonella
Maribacter
Marinobacter
Marinomonas
Marivita
Mesonia
Micrococcus
Nitrosomonas
Oleispira
Photobacterium
Pseudoalteromonas
Pseudomonas
Pseudorhodobacter
Psychrobacter
Rubritalea
Salinisphaera
Staphylococcus
Tenacibaculum
Vibrio
Winogradskyella

Figure 3.4. (A) Relative abundance of phylum level assignments obtained from gill mucus samples. Samples are grouped by timepoint in longitudinal fashion (each bar *n*=3). (B) Alluvial plot of genus level assignments from the top 100 most prevalent ASVs in the study. These data indicate the bacterial community was likely in a dynamic state, which changed rapidly over a short-term period, characterised by large changes in abundance between dominant taxa.

Microbiome profiling of water

In addition to profiling the microbial community of the gills, we also examined the microbial community of the water in which the fish reside. Taxonomically, the dominant tank water derived taxa at the genus level include *Alteromonas*, *Pseudoalteromonas*, *Crocinitomix*, *Tenacibaculum* and *Winogradskyella* (**Figure S3.3**). Overall, many ASVs were common between samples obtained from the gill mucus and the tank environment. The FEAST package was used to determine the source origin of the gill mucus samples, by assessing the contribution from tank water microbiota. A large proportion of tank water-based bacteria were present on the gill for all bath (**Figure 3.5A**) and feed (**Figure 3.5B**) groups. FEAST demonstrated that tank water contributed a significantly higher proportion of the gill mucus community post treatment with hydrogen peroxide. Both H_2O_2 FW and H_2O_2 SW directly post bath and at 1-day sample points were significantly influenced by tank water communities (t-test, $p<0.001$). Conversely, in-feed OTC had the lowest contribution of tank water-based bacteria on the gill surface, which decreased over time.

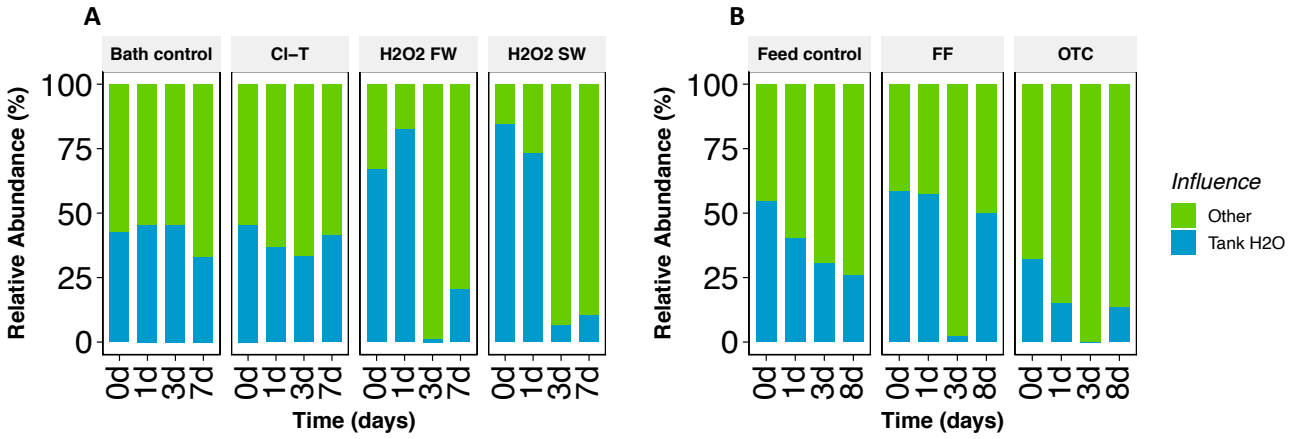

Figure 3.5. Proportions of bacterial sources obtained in gill mucus samples in percent relative abundance. Core bacterial microbiota originating from tank water derived origins (blue) are shown comparative to other sources (green).

3.5. Discussion

The bacterial community upon gill surfaces provide protection to the host by maintaining a diverse range of taxa which deter against pathogenic opportunist microbes. The beneficial action of commensal microbiota is integral to the non-specific immunity of fish species, assisting in the

overall defence against infection and disease. In aquaculture, and other primary production industries, the control of disease sometimes involves therapeutic treatments to be administered to animals to prevent substantive stock losses. However, the residual effect that these treatments have on commensal microbiota and thus barrier health is not fully understood. The antibacterial treatment options used in this study dramatically reduced bacterial counts derived from the anterior holobranch. Interestingly, this effect was relatively brief for fish treated either with dietary antibiotics or a bath immersion. A rapid increase in bacterial abundance following bath treatments was observed, with an increase in culturable colonies at the 1- and 6-hour timepoints, which was generally lower than the bath control samples. Very low numbers of bacterial colonies detected upon the gills treated with OTC and FF persisted slightly longer, with some evidence of recolonisation at 1-day post treatment and peaking at day 3. This was a similar result to that observed by Carlson et al. (2015), where rifampicin treated Gambusia were rapidly recolonised by 2.6 days post-treatment. Bacterial counts taken in this study however were limited to culturable heterotrophic species, and therefore may not entirely reflect total richness or diversity. The rapid enumeration kit protocol employed (3M Petrifilm™) is optimised to grow aerobic heterotrophic bacteria at this temperature as per the AOAC official standard (Australian Government (DAWE), 2021). It is possible that colony counts would have varied when applying different culture conditions (i.e. temperature), however treating the samples identically ensured that all trends are relative to one another in this project, and were optimised and reproducible with the commercial kit.

Nonetheless, this result does demonstrate that the culturable bacterial load can be significantly lowered using antibacterial treatments. Fish that underwent a sham treatment of filtered seawater displayed lower viable counts compared to unbathed control fish that were not fed antibiotics. It is possible that multiple netting and handling events during the immersion bath process may have impacted upon the gill bacterial community. A study completed by Minniti et al. (2017) demonstrated that the Atlantic salmon skin microbiome was greatly altered after fish were netted from a holding tank and their skin bacterial community differed markedly to unhandled fish for at least 24 hours. Handling fish induces an acute stress response in salmonids (Demers and Bayne, 1997), leading to increased ventilatory action and shedding of gill mucus (Roberts and Powell, 2005a), which may reduce bacterial load within the mucosa. Subsequent post-transfer into a tank of ozonated and filtered seawater may also be attributable to the lower overall $CFU.mL^{-1}$ counts (and slower recruitment) than what was recorded in the in-feed treatment groups, where handling had only occurred 10 days prior for that cohort. Irrespective of handling,

the overall effect of antimicrobial treatment resulted in more marked reductions in culturable colonies in fish gill mucus.

Quantitative PCR of the fish gill mucus in this study also demonstrated that there was a brief but significant reduction of 16S rRNA gene copies noted within the OTC and to lesser extent the FF antibiotic treatments at the completion of the ten-day course. Despite being a very different assessment technique to the aerobic count plates for evaluating bacterial load, there appears to be some agreement between results. Previous studies have correlated 16S rDNA assays with reasonable agreement to colony count methodologies (Bach et al., 2002), a result that is logical given the high proportion of aerobic heterotrophic bacteria present in aquatic environs (Cole et al., 1988). Results in the current study demonstrate that the sensitivity of both methodologies are sufficient to capture differences in the bacterial load on the gill surface after antimicrobial treatment in antibiotic fed fish. The interpretation of SYBR green 16S qPCR data for immersion bath groups appeared to remain relatively static in comparison. A possible limitation of this assay for oxidative bath treatments is an inability to distinguish between bacterial DNA that originates from killed or live cells at the time of sampling. It is possible that a reduction in 16S gene abundance may not be observed in the oxidative bath treatments, but the treatments may have still had a significant bactericidal effect. A process such as screening samples with propidium-monoazide (PMA) to remove DNA from lysed cells and measure only live bacterial DNA would be useful to attain further accuracy in future studies, as demonstrated in previous studies (Li et al., 2017). Shannon diversity indices also reflected the post-treatment results observed in the count plate and qPCR data for the antibiotic groups. Both OTC and FF began at a very low Shannon index, which gradually increased throughout the trial period, presumably as bacteria were able to recolonise the gill surface. Richness of the bacterial communities were largely unchanged throughout this early post-treatment period. It may be possible that taxa that were naturally resistant to specific antimicrobial treatments were able to proliferate easily with a lack of competition and substantial nutrient resource (Noga, 2010).

Taxonomically, largescale changes in the bacterial community were noted immediately after bath treatments. This indicates that the composition of the bacterial community was likely impacted by the treatment applications. Most groups appeared to share a low number of dominant taxa (predominantly *Cutibacterium* and *Rubritalea*) from 0-6 hours, but this began to diverge into more disparate communities. The taxonomic analysis did however show a significant shift in phylum and genus from the initial samples and the bath control fish. Interestingly, the movement of fish from freshwater back to marine water in the H_2O_2 FW group resulted in total removal of *Rubritalea*, which colonised again one day post-bath. This is not surprising given that the

Rubritaleaceae are generally psychrophilic marine based bacteria (Song et al., 2019), however the function of this taxon may require more investigation as a coloniser of the gill surface in prior studies (Schmidt et al., 2017; Slinger et al., 2020b; Wilkes Walburn et al., 2019). This taxon appeared to colonise the gill mucus in the highest proportion during both antibiotic treated groups. Source-tracking analysis (FEAST) compared the gill mucus community to the tank water sources collected in this study to determine the proportion of the gill community that is directly influenced by the water environment. Commonality between the 100 most abundant ASVs derived from gill mucus and tank water was very high. The unique species found on the gill surface only related to several known nitrifying taxa (*Marivita*, *Nitrotoga* and *Nitrosomonas*) (Dang et al., 2017; Yoon et al., 2013), along with chemoautotrophs which may occupy favourable niche habitats on the gill surface (e.g. *Marinomonas*, *Pseudorhodobacter* and *Micrococcus*) (Nierychlo et al., 2020). Interestingly, this analysis demonstrates that fish bathed in hydrogen peroxide (both freshwater and saltwater) were initially more likely to recruit gill microbiota from the source tank water, and lacked the enrichment from these functional gill-based taxa. The dominance of tank water sourced gill bacteria was reduced 3 days post-treatment, but suggested that gill mucus recolonisation may at least initially be reflective of microbiota from the external milieu. Water samples obtained at 4 timepoints from experimental tanks were largely consistent, but showed a significant group effect between OTC and Cl-T tank water. This may be due to the poor oral bioavailability of oxytetracycline in marine fish, where complexation is likely responsible for a lack of effective absorption when given as medicated feed in seawater. It is thought that around 90% of the drug passes into the receiving environment in the form of uneaten feed, feaces and urine (Noga, 2010).

Oral administered antibiotic treatments are common practice in aquaculture, where feed is coated with an emulsion of the required drug. Treatments of broad-spectrum antibiotics have been known to remove a significant proportion of the host commensal community, which can cause a range of detrimental effects including increased disease susceptibility (Gupta et al., 2019; Rosado et al., 2019). Studies within mammalian biology have demonstrated that broad spectrum antibiotic application has significant impacts on the host microbiota. In mice, a distinct decrease in species diversity and subsequently higher rates of pathogen colonisation have been observed (Sekirov et al., 2008). Channel catfish (*Ictalurus punctatus*) treated with in-feed florfenicol at 20 mg.kg demonstrated a decrease in diversity compared to non-treated fish, and a significant dysbiosis dominated by the genus *Plesiomonas* for 10 days post treatment (Wang et al., 2019). Some consideration has been given to mitigating the effects of antibiotic usage with an addition of a commensal or functional probiotic. For example, Schmidt et al. (2017) demonstrated that

Phaeobacter inhibens S4Sm and *Bacillus pumilus* RI06-95Sm could effectively colonise fish gills and mitigate the impacts of antibiotic usage post challenge with *Vibrio anguillarum*.

An increase in known pathogen-associated bacteria was observed after treatment in this study, although it appears several taxa are present in low numbers as part of a normal bacterial community. Taxa including *Vibrio* and *Pseudoalteromonas* were most abundant toward the end of the post-treatment period for OTC, FF and bathing in H_2O_2 with saltwater. These taxa, in combination with *Tenacibaculum*, *Staphylococcus*, *Aliivibrio*, *Pseudomonas* and *Photobacterium* made up a high proportion of the 100 most prevalent ASVs detected in this study. This assemblage of known pathogenic microbiota is known as the 'pathobiome', with proliferation of this clade leading to negative impacts to the host by promoting multifocal health issues (Bass et al., 2019; Sweet and Bulling, 2017). These data indicate that antimicrobial treatment and associated husbandry stressors of apparently healthy stock could potentially lead to an increase in abundance of potentially harmful clades of bacteria. It is known that pathogenic threats can be effectively neutralised by a functioning and diverse commensal bacterial layer (Cabillon and Lazado, 2019). The reduction in commensal bacteria from antimicrobial treatment may have resulted in nutrient rich areas of low bacterial density or mucus layer coverage where infiltration of such opportunistic bacteria could occur. It is possible that branchial gill damage from the various treatment options may have removed or partially impacted the mucosal layer initially (Bass and Heath, 1977), which may have also played a role in the subsequent colonisation. Both antibiotics used in the current study have been observed to cause immunosuppressive effects to the specific and innate salmonid immune systems (Enis Yonar et al., 2011; Lundén et al., 1999; Noga, 2010). Similarly, the oxidative action of both chloramine-t and hydrogen peroxide are known to cause innate immune suppression in Atlantic salmon and rainbow trout (Vera and Migaud, 2016; Yavuzcan Yildiz et al., 2009). Such suppression to the immune function of fish gills may provide scope for pathogenic species to more readily colonise these areas, increasing host susceptibility to infection. Such host susceptibility extends to the terrestrial environment, where honey bees exposed to the herbicide glyphosate suffer microbial imbalances to the gut microbiome. Animals challenged with the known pathogen *Serratia marcescens* suffer higher mortality as a result of this antimicrobial action (Motta et al., 2018). Further substantiation is required to determine if direct impacts of the antimicrobial treatment, possible immunosuppression on the host (from treatment), or the absence of the commensal microbiota after microbial reduction/alteration can influence pathogenic colonisation of the gill and susceptibility.

In this study we examined several antimicrobial treatment applications that successfully reduced culturable gill bacteria, and caused significant post-treatment impacts on branchial bacteriomic diversity and taxonomic composition. The results from this study support previous research suggesting that antimicrobial treatments may have significant and lasting effects on the composition of branchial microbiota. Thus, it is concluded that these identified antimicrobial treatments are potential candidates for future usage in branchial microbial studies investigating the phenomenon of dysbiosis, how this may impact upon microbial gill disease or more broadly the role of commensal microbiota in fish health.

Chapter 4. The effect of antimicrobial treatment upon the gill bacteriome of Atlantic salmon (*Salmo salar* L.) and progression of amoebic gill disease (AGD) *in vivo*

This chapter is a verbatim reproduction from the following published paper:
Slinger, J., Adams, M.B., Stratford, C.N., Rigby, M., Wynne, J.W., 2021. The Effect of Antimicrobial Treatment upon the Gill Bacteriome of Atlantic Salmon (*Salmo salar* L.) and Progression of Amoebic Gill Disease (AGD) *In Vivo*. Microorganisms 9, 987.
https://doi.org/10.3390/microorganisms9050987

4.1. Abstract

Branchial surfaces of finfish species contain a microbial layer rich in commensal bacteria which can provide protection through competitive colonisation and production of antimicrobial products. Upon disturbance or compromise, pathogenic microbiota may opportunistically infiltrate this protective barrier and initiate disease. Amoebic gill disease (AGD) is a globally significant health condition affecting salmonid mariculture. The current study examined whether altering the diversity and/or abundance of branchial bacteria could influence the development of experimentally induced AGD. Here we challenged Atlantic salmon with *Neoparamoeba perurans* in a number of scenarios where the bacterial community on the gill was altered or in a state of instability. Administration of oxytetracycline (in-feed) and chloramine-t (immersion bath) significantly altered the bacterial load and diversity of bacterial taxa upon the gill surface, and shifted the community profile appreciably. AGD severity was marginally higher in fish previously subjected to chloramine-t treatment following 21 days post-challenge. This research suggests that AGD progression and severity was not clearly linked to specific bacterial taxa present in these systems. However, we identified AGD associated taxa including known pathogenic genus (*Aliivibrio*, *Tenacibaculum* and *Pseudomonas*) which increased in abundance as AGD progressed. Elucidation of a potential role for these bacterial taxa in AGD development is warranted.

4.2. Introduction

Teleost fish mucosa is a functionally important tissue constructed of macromolecules and polymers containing numerous enzymes and protective peptides (de Bruijn et al., 2018; Gómez and Balcázar, 2008). The mucosa forms a structural medium that facilitates colonisation of beneficial microbiota which play a key role in the health and function of the animal (Naik et al., 2012; Reverter et al., 2018). The bacterial community which colonise the mucosal layer contains both transient and resident taxa which utilise available resources (van Kessel et al., 2016) and perform key roles such as competitive exclusion or inhibition of unwanted pathogens (Cabillon and Lazado, 2019; Kelly and Salinas, 2017; Minniti et al., 2017).

Dysbiosis is a community level imbalance of microbial taxa, typically characterised by a disturbance or perturbation (Moya and Ferrer, 2016; Romero et al., 2014). Environmental stressors or disease can lead to a dysbiosis of mucosal bacterial communities in an aquatic setting (Egan and Gardiner, 2016). For example, rapid temperature reduction and air exposure applied to the late egg developmental stages significantly affected the gut and skin community of larval Atlantic salmon (*Salmo salar*) (Webster et al., 2020). Similarly, transfer of Atlantic salmon smolt from freshwater to seawater caused an appreciable transition of the microbiota occupying the skin mucus (Lokesh and Kiron, 2016). Significant loss of bacterial richness and a destabilization of the skin community composition was reported from Atlantic salmon infected with sea lice (Llewellyn et al., 2017). *Aeromonas salmonicida* dominated the intestinal microbiota in furunculosis affected largemouth bronze gudgeon (*Coreius guichenoti*, exhibiting a significant dysbiosis compared to unaffected fish (Li et al., 2016). A largely unexplored area of microbial dysbiosis is the susceptibility of external barriers to pathogen outbreaks following bacterial dysbioses.

Disease treatment or prevention via antimicrobial compounds is often crucial to mitigate stock losses in the event of bacterial or fungal infections, including prominent salmonid diseases such as sea lice (*Lepeophtheirus salmonis*), yellow mouth (*Tenacibaculum* spp.) and bacterial gill disease (*Flavobacterium* spp.) (Bowker et al., 2008; Kiemer and Black, 1997; Wynne et al., 2020b). Typically, these treatments are 'broad spectrum' in nature, and can contribute to imbalances of the microbial consortia (de Bruijn et al., 2018).

Amoebic gill disease (AGD) is a proliferative gill condition predominantly affecting salmonid mariculture. The causative agent, *Neoparamoeba perurans* is a free-living marine amoeba species which attaches to gill lamellae eliciting focal necrosis, oedema, inflammation and hyperplasia of the gill epithelium (Adams and Nowak, 2003; Crosbie et al., 2012; Munday, 1986; Roubal et al., 1989; Wiik-Nielsen et al., 2016; Young et al., 2007) leading to physiological disturbance and mortality if untreated (Chang et al., 2019; Harris et al., 2005; Leef et al., 2005; Munday et al.,

2001; Powell et al., 2008). *N. perurans* is ubiquitously distributed throughout many salmon production areas (Bridle et al., 2010; Wright et al., 2015), comprising part of the microbial community within the external milieu alongside numerous other marine microbes (e.g., viruses, fungi, bacteria, other protozoa). The external surfaces of finfish species such as Atlantic salmon are in constant contact with these pathogens and the potential threat they pose.

While the primary pathogenic role of *N. perurans* in AGD has been unequivocally confirmed via Koch's postulates (Crosbie et al., 2012), knowledge gaps exist regarding potential relationships with other microbes. Initiation of AGD occurs via adherence of *N. perurans* to the gill mucosa, where commensal bacteria may be present (Adams et al., 2004; Embar-Gopinath et al., 2005a; Roubal et al., 1989). Given that amoebae can utilise bacteria as a feed source or coexist in a symbiotic arrangement it remains possible that bacterial taxa may have a role in the progression of AGD (MacPhail et al., 2021; Slinger et al., 2020a). A limited number of previous studies have suggested that particular bacteria are associated with amoebic branchialitis and/or may affect the onset and severity of this condition. *Pseudomonas* sp. were observed within and around trophozoites of *Paramoeba* sp. isolated from AGD affected Atlantic salmon with small round bacteria observed in histological gill sections from the corresponding fish (Roubal et al., 1989). A culture-independent study of gill bacteria (Bowman and Nowak, 2004) demonstrated a small number of AGD affected fish in both field and laboratory scenarios were dominated by a phylotype assigned to *Psychroserpens* sp. The authors proposed this species was a potential opportunistic pathogen associated with AGD. A subsequent culture-dependent study observed the genera *Winogradskyella* and *Staphylococcus* in association with AGD affected Atlantic salmon (Embar-Gopinath, 2006) proposing a similar link or association of this bacteria with AGD. A follow-up study found a higher percentage of lesion affected gill filaments in Atlantic salmon following colonisation *Winogradskyella* sp. and challenge with *Neoparamoeba sp.* (Embar-Gopinath et al., 2005a). Recently, it was confirmed that the bacterial community associated with AGD affected gill lesions can be dominated by pathogenic species such as *Tenacibaculum dicentrarchi* (Slinger et al., 2020a). The pathogenicity of other disease-causing amoebae species can be affected by bacterial presence. For example, *Entamoeba histolytica,* responsible for mammalian intestinal enteritis were co-cultured with known pathogenic bacteria increasing the rate of adhesion and cytopathic effect to host cell lines (Galván-Moroyoqui et al., 2008). Amoeba keratitis of the human eye caused by numerous *Acanthamoeba sp.* has been strongly linked to several bacterial co-factors, including *Corynebacterium xerosis* (Badenoch et al., 1990). The timing and nature of interactions from pathogenic amoebae species as an opportunistic or synergistic

process, and the subsequent impact on the commensal resident microbiota is not yet fully understood.

Identification of bacterial taxa that colonise the gills during an amoebic infection may provide further understanding of interactions between amoebae and bacterial species, and whether these interactions play a role in onset and progression of AGD. Therefore, the aim of the present study was to investigate the impact upon experimentally induced AGD progression by altering the bacterial load upon the gills prior to infection by *N. perurans*. We also examined whether bacterial community structure and diversity is altered by experimental infection and disease caused by *N. perurans*.

4.3. Materials and Methods

All animal activities relating to fish use in this trial were approved by the CSIRO QLD Animal Ethics Committee under the permit numbers CQAEC 2017–35 and CQAEC 2018–18.

4.3.1. Fish source and husbandry

Atlantic salmon fingerlings (all female, diploid) obtained from the Rookwood Road hatchery in Ranelagh, Tasmania at approximately 8 g mean weight were transferred to a freshwater recirculating aquaculture system (RAS) (5000 L) at the Bribie Island Research Centre. Fry were ongrown for approximately 8 months, before being exposed to 24 h light (3200 lumen) for a period of 5 weeks, after which the water salinity was raised from 3 ppt to approximately 36 ppt. Fish were acclimated at this salinity for approximately 4 weeks prior to the trial commencing. Water temperature was held at 15 °C ± 0.5, dissolved oxygen at 90–110% sat, TA-N < 0.50 $mg{\cdot}L^{-1}$, and salinity 35–36 ppt.

4.3.2. Experimental design, procedures and maintenance

Ninety fish were haphazardly selected by dip-net and transferred to an independent 500 L tank and fed oxytetracycline hydrochloride (OTC) administered in-feed for 10 days (CCD, Tamworth, NSW, Australia). Feed pellets (3 mm Spectra, Skretting Pty Ltd., Cambridge, TAS, Australia) were vacuum coated at 350 P.S.I for 5 min with a 2% fish oil-based emulsion containing 79 $mg{\cdot}kg^{-1}$ OTC powder for every kg of feed, such that a 1% bodyweight ration would equate to a dosage of 79 $mg{\cdot}kg^{-1}$of fish biomass. OTC coated pellets were stored at −20 °C, with the required ration taken from the freezer to be loaded into the autofeeders each morning.

4.3.3. Pre-challenge with antimicrobial treatments

At the beginning of the trial period Atlantic salmon post-smolt (350 ± 1 g) were anaesthetized using 17 $mg{\cdot}L^{-1}$ AQUI-S® (Aqui-S Ltd., Lower Hutt, NZ, New Zealand), and exposed to a

combination of bathing steps as depicted in **Figure 4.1**, dependent on the experimental treatment (n = 30 fish per bath replicate; 90 fish total per treatment).

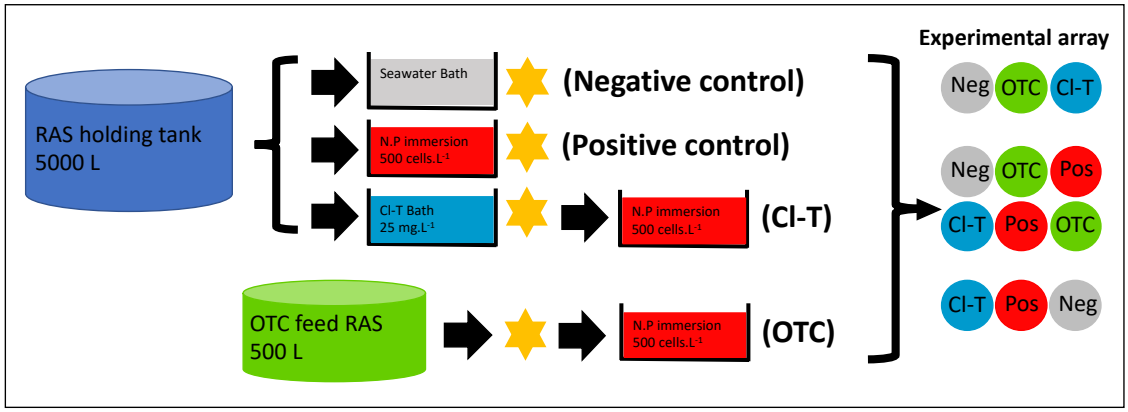

Figure 4.1. Flow diagram of experimental treatment preparation used in this study, showing treatment or challenge steps prior to stocking into experimental array (each treatment group was replicated three times). Star shape depicts when initial (Time 0) gill mucus sampling of 5 fish per tank (15 fish per treatment group) was completed.

Fish selected for the chloramine-trihydrate (Cl-T) therapeutic bath treatment were netted out into a disinfected plastic tub, containing a homogenized solution with 300 L of filtered seawater (15 °C) and 7.5 g of chloramine-trihydrate powder (Sigma-Aldrich, St. Louis, MO, USA) under constant aeration (~90–100% Sat.). This nominal dose of 25 mg·L^{-1} was verified by measuring (total chlorine – free chlorine) x 3.97 (Y.S.I 9500 photometer), and applied for a period of 1 h, as per the Food and Drug Administration (FDA) recommendations for therapeutic application in aquaculture.

After antimicrobial treatment, fish were stocked into an array of 12 identical 500 L tanks (n = 25 per tank). Array tanks assigned to the AGD affected (positive control, Cl-T) and AGD naïve (negative control) treatments were offered a feed ration of 1% bodyweight daily (3 mm Skretting Spectrum pellet) via autofeeders (Arvotec wolf controller, Arvotec-Oy, Huutokoski, Finland). Fish from the OTC fed holding RAS were also stocked into the flow through system and maintained the OTC treatment dose of 79 mg·kg^{-1} for the experimental duration (21 days). Experimental tanks were supplied with flowthrough seawater (~6 L·min^{-1}) that was filtered (20 µm), ozonated (100 gO_3·h^{-1}), UV sterilised (80 mJ·cm^2) and chilled to ~15 °C. Fish were monitored via constant data logging of water temperature and dissolved oxygen (Oxyguard Pacific, Farum, Denmark) and photoperiod was maintained at 12^L:12^D. Daily maintenance was carried out in the form of observing fish behaviour for overt signs of AGD including listlessness and excessive opercular movement, as well as cleaning tank systems and collection of any

uneaten feed at the conclusion of the autofeeder activity period. This collected feed was retained into a mesh sieve, where a pellet count could be taken to ensure normal feed rates occurred.

4.3.4. Sampling strategy

Gill mucus collection

Sampling of fish gill mucus was carried out directly following antimicrobial treatment (**Figure 4.1**), at the 11 dpi midpoint sample (5 fish per tank), and again at 21 dpi (15 fish per tank). At each sampling timepoint five individual fish from each tank (15 fish per treatment) were haphazardly dip netted from the tank and euthanised (by immersion in 100 mg·L^{-1} AQUI-S®). A sample of mucus was taken by swabbing the surface of all anterior and posterior hemibranchs from the entire left-hand side of the gill basket (8 surfaces). This was achieved by rotating a sterile cotton swab (Westlabs, Ballarat, VIC, Australia) three times on each of the hemibranch surfaces. Swabs were then transferred to a 1.5 ml screw cap tube containing 1 ml of RNAlater solution, and stored at 4 °C for 24 h before being frozen and stored at −80 °C until further processing.

Tank water samples

Representative water samples were obtained from all water sources used during trial setup in triplicate, along with each experimental tank at the 11- and 21-day post inoculation (dpi) sampling points. Briefly, ~700 ml of water was collected in triplicate disinfected HDPE containers by placing the container approximately 10 cm subsurface and opening the lid. Each of these samples were then filtered across a 0.22 μm Sterivex filter membrane (Millipore, Burlington, MA, USA) using a peristaltic pump unit (Lachat Instruments, Milwaukee, WI, USA), and disinfected tubing with luerlok fittings. The filter chamber was then flooded with approximately 3.5 ml of RNAlater solution and stored at 4 °C prior to DNA extraction.

4.3.5. DNA extraction and purification

Gill mucus

All gill mucus samples underwent DNA extraction using the DNeasy PowerSoil kit (Qiagen, Hilden, Germany). RNAlater preserved swabs were placed into a Tissue Lyser (Qiagen, Hilden, Germany) for 10 min at a frequency setting of 15.0 Hz before pulse centrifuging of each individual tube. The swabs were removed using a sterile forceps, taking care not to cross-contaminate samples, and placed into a labelled 2 ml tube. The remaining RNAlater was then spun down at 17,000× *g* for 10 min in order to form a visible pellet. RNAlater was then pipetted

to waste, taking care not to dislodge the pellet. Both the pellet and the swab were stored at −80 °C until processing. To process, 60 μL of Solution C1 was added to the PowerBead tube, mixed by pipetting up and down, then 200 μL was removed and used to collect the thawed pellet, with all liquid returned to the PowerBead tube along with the corresponding swab. The PowerBead tube containing the pellet and swab was then sharply tapped upside down on the benchtop to ensure the beads moved freely around the swab. Samples were then vortexed horizontally using a vortex adapter tube holder at maximum speed for 20 min. Extraction steps were completed according to the manufacturer's instructions, with an elution volume of 50 uL and genomic DNA was assessed for yield and quality using a Nanodrop ND-1000 spectrophotometer (Life Technologies, Carlsbad, CA, USA). Samples were stored at −20 °C until downstream use.

Tank water

Bacterial DNA was extracted from 0.22 um Sterivex (Millipore, Burlington, MA, USA) water filter units. Filter samples were extracted using the DNeasy PowerWater Sterivex kit (Qiagen, Hilden, Germany), as per manufacturers protocols. Genomic DNA quality and concentration was verified using Nanodrop ND-1000 spectrophotometer (Life Technologies, Carlsbad, CA, USA). DNA from triplicate pooled samples were combined prior to storage. Samples were stored at −20 °C until sequencing.

4.3.6. Challenge with *Neoparamoeba perurans* and AGD assessment

An immersion bath containing *Neoparamoeba perurans* trophozoites in seawater was used to challenge fish in the AGD positive control, Cl-T and OTC groups (**Figure 4.1**). A dedicated 'constant infection tank' (CIT) consisting of a 2000 L RAS containing Atlantic salmon smolt to passage wild-type *N. perurans* was used for this experiment. Firstly, water from this CIT system (1 L) was collected using sterile 50 ml tubes and concentrated down by centrifuge at 4000× *g* to a final volume of 10 mL. Counts of this subsample on a hemocytometer ($n = 10$) were then used to enumerate the concentration of amoebae cells per liter within the CIT. After estimating the amoebae load within the infection system, a sufficient volume of well-homogenized water was transferred to a disinfected 500 L tub, and made up to 100 L with filtered seawater to achieve a cell concentration of 500 cells·L^{-1}. Fish were netted into this bath and maintained for a period of 1 h, with supplemental aeration provided. The AGD naïve treatment group (negative control) underwent a sham immersion bath containing only filtered seawater. All fish were hand netted into the experimental array after this process, and the *N. perurans* bath setup step was repeated with each replicate to account for amoebae cells lost from the bath via adherence to the gill.

Gill score assessment

Following euthanasia but prior to sample collection the gross gill score was recorded for each sampled fish. AGD gill scoring was performed as described by (Taylor et al., 2009), where all 16 arches are visually assessed for white multifocal mucoid patches. A score between 0 (no visible AGD) and 5 (severe AGD) was then assigned to each individual fish to give a gill index per tank and treatment.

Gill histopathology

The gill basket from each fish was excised using sterile micro scissors and placed into a specimen jar containing seawater Davidson's fixative, where each holobranch was dissected individually and all 16 arch surfaces were photographed using a lightbox and SLR camera (Canon EOS 7D), before transfer to 70% EtOH after 48 h. Subsequently, the third arch from the right-hand side (R3) was excised from the gill basket, and routinely processed, infiltrated and embedded in paraffin. Samples were sectioned (5 μm) from the anterior hemibranch surface using a Microm microtome (Thermo Scientific, Waltham, MA, USA) and placed onto glass slides. The slides were then stained (H&E), cover-slipped and examined under a Leica DM1000 light microscope (Leica Microsystems, Wetzlar, Germany). The hemibranch section was assessed for the proportion of filaments with hyperplastic gill lesions and the percentage of lesions with *N. perurans* present.

Quantitative PCR assay

DNA from gill mucus samples (obtained in 4.3.5) were analysed using a TaqMan® qPCR targeting the 18 S rRNA gene sequence of *N. perurans* generating an amplicon of 70 bp (**Table S4.1**). The salmon elongation factor gene (Ef1α) described in (Bruno et al., 2007) was used as the reference gene in this assay, amplifying a 66 bp fragment. Each real-time PCR reaction mixture contained 4 μL template, 5 μL TaqMan® Fast Advanced Master Mix (Applied Biosystems) with a final reaction volume of 10 μL. The thermal profile of the real-time PCR program consisted of 2 min at 50 °C, 2 min at 95 °C, followed by 45 cycles of 1 sec at 95 °C and 20 s at 56 °C in an QuantStudio™ Real-Time PCR instrument (Applied Biosystems, Foster City, CA, USA). Each plate included a *N. perurans* positive and negative control, as well as a PCR 'no template' control in triplicate for both the target and reference gene. Samples were run in triplicate for *N. perurans* and duplicate for salmonid elongation factor-1α (Ef1α). Analysis of the real-time data involved setting the threshold across all plates for *N. perurans* and Ef1α at 0.1 and 0.04, respectively.

Quantitative PCR data was assessed using the delta-delta Ct ($2^{-\Delta\Delta Ct}$) method derived by (Livak and Schmittgen, 2001). Data were estimated by comparing the ratio of ΔCt of the gene of interest (*N. perurans* 18 S)-ΔCt of the housekeeping gene (salmon Ef1α) for each gill swab sample. After this, ΔΔCt was calculated by measuring the ΔCt (treated fish sample)-ΔCt (untreated fish mean) and the relative fold gene abundance change was calculated by transforming data ($2^{-\Delta\Delta Ct}$).

4.3.7. Gill bacteriome assessment

Branchial bacteria counts

A gill mucus swab of the right-hand side anterior hemibranch (R1) was collected from 5 fish per tank (n = 15 per treatment) to estimate culturable bacterial loads on the gill. Samples were taken via a sterile cotton swab (Westlabs, Ballarat, VIC, Australia) of the hemibranch (three rotations along the length of the arch) and placing the swab into 1 ml of filtered, autoclaved seawater in a 1.5 ml tube. Each tube was then agitated via vortex for 15 s, before a 500 L aliquot was pipetted onto individual Petri-film® aerobic count films (3M, Saint Paul, MN, USA) and incubated at 35 ± 0.1 °C for 48 h. After incubation discrete colonies were visually counted within the film grid area and recorded (data were calculated as $CFU \cdot mL^{-1}$).

16S rRNA amplicon sequencing

DNA obtained from gill mucus and tank water (4.3.5) underwent amplicon sequencing, targeting the V1–V3 hypervariable region of the 16 S rRNA gene. This was prepared via a "2-step" PCR submission process, using the Illumina recommended adapter-fused overhangs applied to the V1–V3 amplicon primers (bold) as shown in **Table S4.2**. A total of 25 cycles were performed with normalised DNA at an average of 10 $ng \cdot \mu L^{-1}$. Samples in this study included a mock positive control (ZymoBIOMICS Microbial Community Standard, Zymo Research), and two negative controls (blank swab process control and blank DNA extraction laboratory control). Sequencing was performed at the University of New South Wales, (Ramaciotti Center for Genomics Sydney, Australia) via an Illumina Miseq platform with 300 base pair (bp) paired end reads.

4.3.8. Bioinformatics pipeline

Raw Illumina amplicon sequencing data files were processed using the open-source software pipeline "Quantitative Insights into Microbial Ecology 2" QIIME2 (Bolyen et al., 2019). Paired end sequences from the forward and reverse reads were merged for each sample and were

denoised using the q2-dada2 plugin (Callahan et al., 2016) with default parameters. Quality control including chimeric sequence removal from the dataset was completed during dada2 processing, along with subsequent removal of host DNA and exclusion of chloroplast and mitochondrial sequences. Amplicon Sequence Variants (ASV's) were classified taxonomically using the classify-sklearn method in the QIIME2 q2-feature-classifier plugin using default parameters (Bokulich et al., 2018). The SILVA 16 S rRNA 99% taxonomy database release 132, (Quast et al., 2012), was used as reference sequences for taxonomic classification.

4.3.9. Statistical analysis

All statistics were performed in R version 3.6.1 (R Core Team, 2019), with QIIME2 artefact files imported using the Qiime2R package (https://github.com/jbisanz/qiime2R, accessed on 5 December 2020). For all statistical analyses, the significant *p*-value was <0.05, except where an adjusted significance is stated. In the amplicon data, obvious contaminant artefact present in the negative control sequences was identified and subsetted from biological samples via the Decontam package (Davis et al., 2018). Samples were rarefied using R package QsRutils (Quensen, 2020) performed on a maximum subsampling depth of 13,460 sequences per sample (**Figure S4.1**). Using the Phyloseq R package (McMurdie and Holmes, 2013) alpha diversities were calculated based on observed ASVs, Shannon diversity and Faith's phylogenetic distance metrics, and the differences between groups were analysed using the non-parametric Kruskal–Wallis and Wilcoxon rank-sum tests. Beta-diversity comparisons were made via NMDS using Bray Curtis pairwise distances. Differences between groups was analysed using PERMANOVA testing from the pairwise Adonis package (Martinez Arbizu, 2019). Differential abundance testing was completed using microbiomeSeq package found in (Ssekagiri et al., 2017), where log-fold change of taxa prevalence at the genus level were compared between groups referenced against the negative control. Genera were considered significant at an adjusted *p*-value (*padj)* <0.01.

Count plate data (bacterial load) were expressed as $CFU \cdot mL^{-1}$ and a log transformation performed. The log-fold CFU data was assessed using a general linear model to test treatment by timepoint. The subsequent significant interaction was further substantiated using a one-way ANOVA and Tukey PSD post hoc testing (*padj* < 0.05). Gill score data (median) was compared using a non-parametric Kruskal–Wallis test, and using a Wilcoxon rank sum test to assess pairwise Treatment and Timepoint interactions. Quantitative PCR data were analysed as log-fold change between treatment groups of the gene of interest (*N. perurans* 18 S) after normalisation against the internal control (salmon Ef1α), log transformed and assessed using two-way ANOVA

with treatment and timepoint as factors (*padj* < 0.05). Histology data for proportion of affected filaments and % of lesions colonised by amoebae was arcsine transformed (to account for non-normality, determined by Shapiro–Wilk testing) prior to statistical analysis. Data were assessed by one-way ANOVA, with subsequent pairwise comparison using a Tukey's HSD test. All figures were produced using the R package ggplot2 (Wickham, 2016).

4.4. Results

At the trial commencement, two mortalities occurred in the Cl-T treatment group, post amoebae exposure (identified as moribund on transfer to experimental tank, and humanely euthanised). Upon further inspection a lower jaw deformity and shortened opercula was identified in both fish. Due to the significant reduction in respiratory efficiency of jaw/opercula deformed fish, it was deduced during necropsy that these individuals may have succumbed to respiratory stress.

4.4.1. Onset and Progression of AGD after Challenge with *N. perurans*

Gross clinical signs of AGD including raised multifocal lesions on the gill surface were visually observed in AGD affected fish in all groups challenged with *N. perurans* (**Figure 4.2B**). Gill score post-inoculation increased from 11 dpi to 21 dpi in all AGD positive groups; OTC (+1.12), Cl-T (+1.05) and positive control (+1.75). At both timepoints, the gill scores of Cl-T and OTC treated fish were similar ($p>0.05$), but the Cl-T and positive infection groups were significantly different($p<0.05$). Small mucoid patches were occasionally observed in unchallenged control fish at both timepoints. The gill index (0.30) remained equivocal at both timepoints but significantly different to all AGD positive groups($p<0.001$).

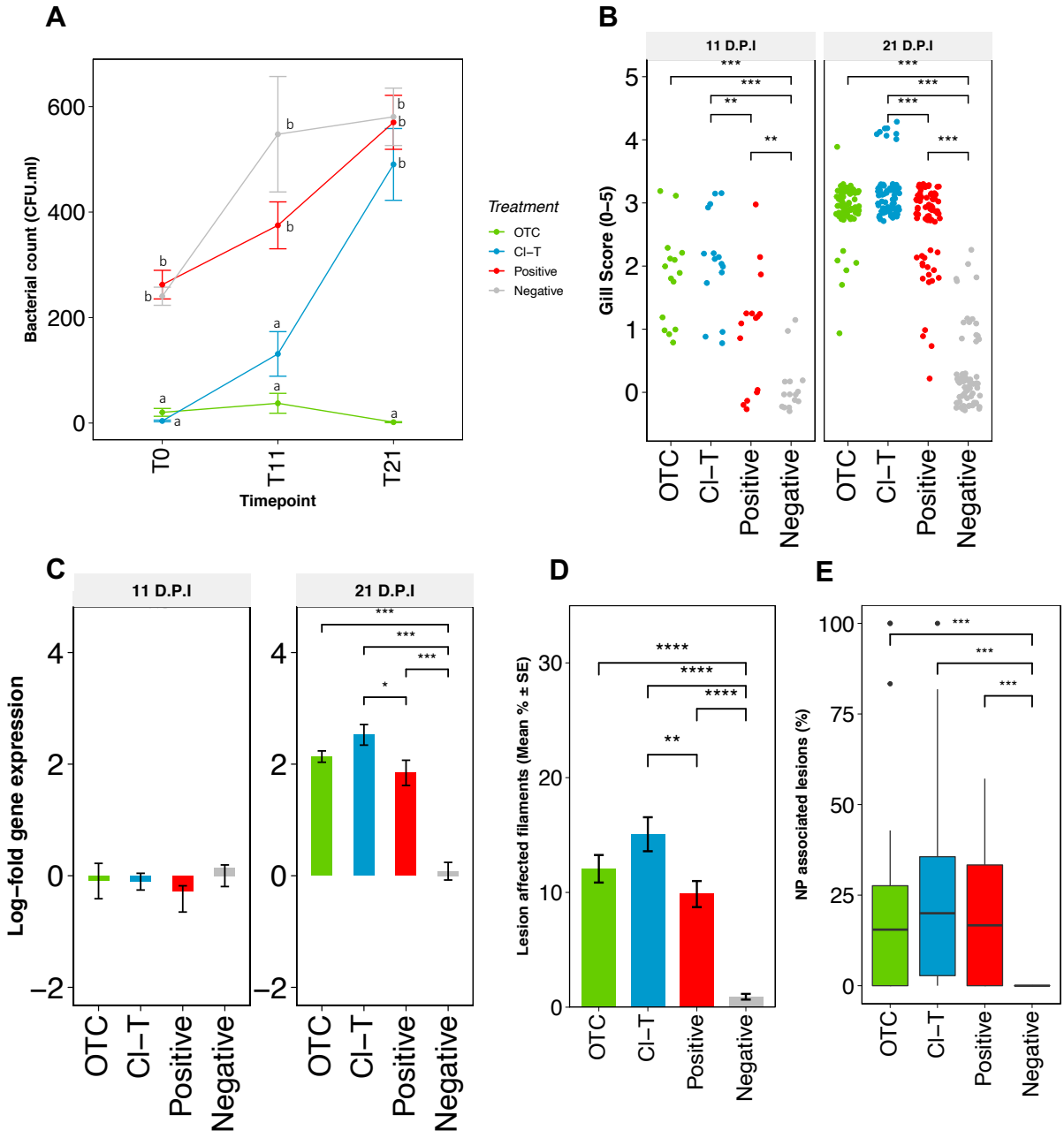

Figure 4.2. (A)-Bacterial counts ± SE obtained from gill mucus swabs derived from the RH1 anterior hemibranch surface. CFU·mL data was log-transformed for statistical analysis; letters indicate significantly different subset groups between all treatments and timepoints (*padj* < 0.05). (B)-Frequency dotplot of visual gill scores obtained from 11 and 21 dpi, with statistical comparison of treatment groups via nonparametric Kruskal Wallis, pairwise statistical differences are presented with asterisks (p<0.01 = **, p < 0.001 = ***). (C)-Relative fold change ± SE (in log2 scale) of *N. perurans* 18 S gene abundance as a function of the reference housekeeping gene (Ef1α) measured by RT-qPCR during amoebic challenge period (11 and 21 dpi). Each bar represents the mean for each experimental replicate (n = 15), statistical differences are presented with asterisks (p<0.05 = *, p < 0.001 = ***). (D)-Histopathological quantitation (n = 30 per treatment) depicting the percentage of lesion affected filaments at 21 dpi with *N. perurans*. Statistical assessment is based on arcsine-transformed data, and presented with asterisks (p<0.05 = *, p < 0.0001 = ****. (E)–Percentage of hyperplasic gill lesions or plaques colonised with amoebae trophozoites, outlying datapoints are represented by black dots, and pair-wise statistical differences are presented with asterisks (p<0.001 = ***).

The log-fold delta-delta (ΔΔCt) change between expression of the 18 S *N. perurans* gene and the salmon Ef1α housekeeping gene demonstrated that the amoebic burden increased throughout the 21 dpi challenge period. When the target gene was standardised to the housekeeping gene of the mean AGD positive control ΔCt these data suggest that the relative *N. perurans* burden was highest within the Cl-T treatment tanks, followed by the OTC and positive AGD groups at 21 dpi (**Figure 4.2C**). There was a statistical difference in qPCR data between the Cl-T and positive control fish ($padj < 0.05$), as well as between the negative non-AGD control and the three AGD exposed groups assessed ($padj < 0.001$ at 21 dpi, **Figure 4.2C**).

At 21 dpi, pathological observations showed typical AGD lesions characterised by multifocal epithelial hyperplasia, lamellae fusion, interlamellar vesical formation and oedema (**Figure 4.3A,B**). *N. perurans* trophozoites with visible cell nuclei were also observed in aggregation along lesion margins (**Figure 4.3B**). Gills from unchallenged fish appeared largely normal (**Figure 4.3C,D**). Small lymphocytic nodules were occasionally observed (1–3 interlamellar units) in all groups. AGD affected gill filaments in the *N. perurans* challenged fish was highest in the Cl-T treated fish (15.06% SE ± 1.48), followed by OTC (12.05% SE ± 1.19) and positive (9.85% SE ± 1.35) groups. The percentage of lesion affected filaments in AGD exposed groups were significantly higher than unchallenged control ($p<0.001$ **Figure 4.2D**). Amoeboid trophozoites were not observed in gill sections from unchallenged fish. Comparisons of AGD exposed fish identified that the Cl-T group was significantly higher than the positive group ($p<0.01$) at 21 dpi. No significant interaction was observed between the OTC group and either the positive control or Cl-T groups. The proportion of lesions colonised by amoebae was slightly higher in Cl-T treated fish (**Figure 4.2E**), but there was no significant interaction between *N. perurans* exposed fish. The unchallenged (negative) control was significantly different from all AGD exposed groups ($p<0.001$).

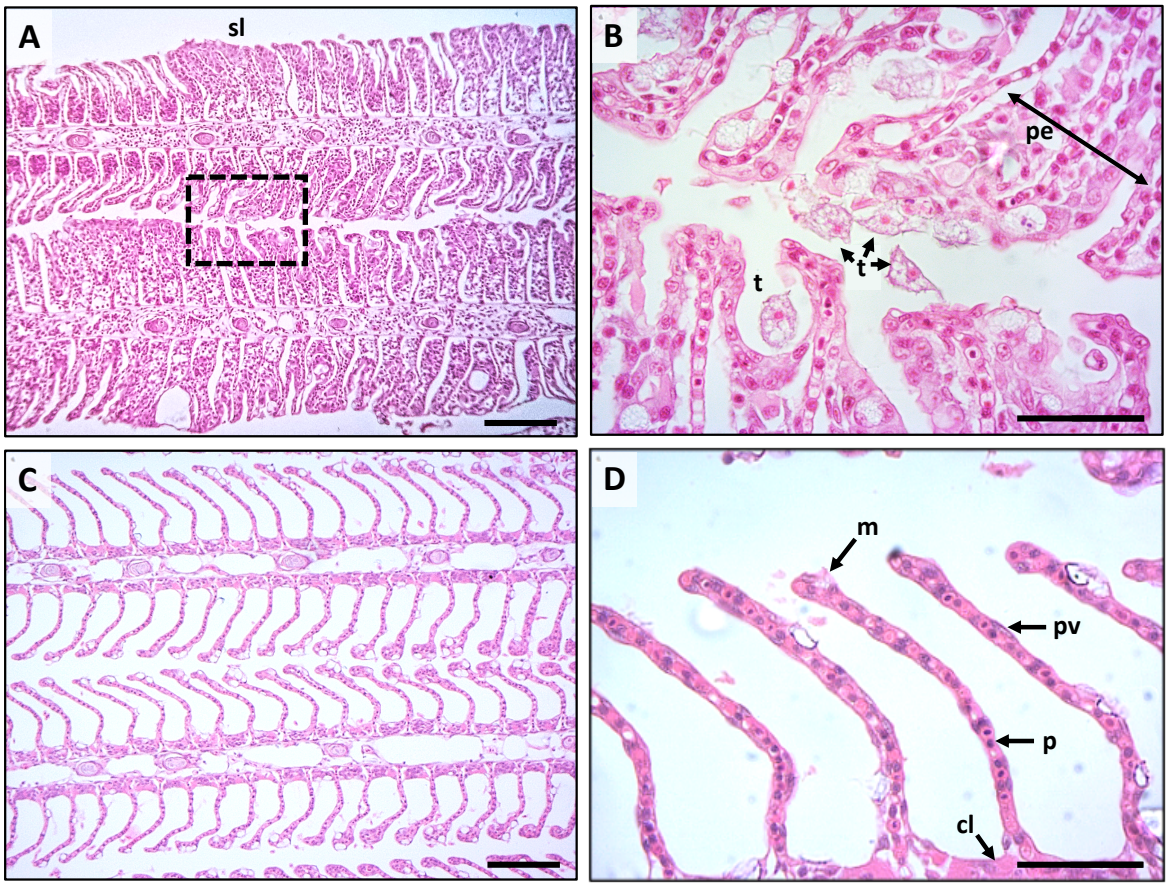

Figure 4.3. Representative histological sections of gill obtained from the experiment; (A) Gill lesion along both margins of primary lamellae, showing extensive fusion of secondary lamellae (sl), scale bar = 50 µm. (B) shows border inset from (A) at 400× magnification, demonstrating *N. perurans* trophozoites with clearly discernable nuclei (t) attached and ad-jacent to areas of proliferating epithelium (pe) scale bar = 100 µm. (C) negative control group, depicting anatomically normal gill morphology, scale bar = 50 µm. (D) Higher magnification of normal healthy gill (400×) showing typical cell types including mucus (m), pavement (pv), pillar (p) and chloride (cl) cells, scale bar = 100 µm.

4.4.2. Gill bacteriomic profiles

Following antibacterial treatment

Culturable bacterial colony counts were significantly reduced in Cl-T directly post treatment, and in OTC throughout the experimental duration (**Figure 4.2A**). Samples from the positive and negative treatment groups were not significantly different from each other, but did increase over time from the 0, 11 and 21 dpi measures. The Cl-T group was significantly lower than the positive and negative groups in the initial timepoint ($F = 7.112$, $df = 2,6$, $p < 0.001$), but was not significantly different at the 21 dpi timepoint.

Concurrent to Neoparamoeba perurans challenge

After processing the *16 S rRNA* gene V1–V3 region sequencing data using QIIME2, an ASV table with 7515 assigned taxa was generated in 218 samples. Bacterial community richness was highest in the OTC treatment fish group at the initial timepoint of the experiment (**Figure 4.4A**). This was significantly different from the 11 dpi and 21 dpi timepoints for this treatment (KW, $p < 0.001$). The Observed ASVs in all other treatments slightly increased from T0 to 21 dpi, with the Cl-T increasing significantly between 11 dpi and 21 (**Figure 4.4A**, KW, $p < 0.01$). In parallel to the observed richness, Shannon diversity (**Figure 4.4B**) in the OTC group was highest at the commencement of the trial, decreasing sharply after this time. Shannon diversity in Cl-T, positive and negative treatment groups stabilised between T0 and 11 dpi, before increasing at 21 dpi. While the Shannon index did increase over time in the negative treatment, it remained largely static in terms of phylogenetic diversity (Faiths PD; **Figure 4.4C**). The AGD positive treatment group increased in phylogenetic diversity over the trial period although this was not significant. The OTC treatment decreased significantly between T0 and both 11 dpi and 21 dpi, whilst the Cl-T group decreased significantly at the mid-point (11 dpi; $p < 0.01$), but was largely unchanged between T0 and 21 dpi (**Figure 4.4C**). Alpha diversity in tank water sampled at 11 dpi and 21 dpi showed a dramatic increase in both observed ASVs and species diversity over time. The Cl-T and OTC tanks represented the highest richness and diversity at the 21 dpi timepoint (**Figure 4.4D**).

Beta diversity was visualised using NMDS ordinations based on Bray Curtis pairwise distances for all fish gill mucus and rearing water samples. PERMANOVA analysis showed that there was a significant interaction between gill mucus communities at each experimental timepoint ($F = 11.9$, df = 2, $p < 0.001$) and antimicrobial treatment group ($F = 6.8$, df = 3, $p < 0.001$). An interaction effect between the two factors, Treatment * Time was also observed ($F = 5.29$, df = 6, $p < 0.001$; **Figure 4.4E**). Pairwise Adonis revealed that there was a high degree of separation between treatment groups, with all treatment groups significantly different at time 0 ($p<0.001$). As the trial progressed the fish gill community tended to converge closer. At day 11 positive and OTC groups were not significantly different, but the Cl-T treated fish were significantly different from all other groups at this timepoint ($p<0.001$). At day 21, the Cl-T and positive groups were not significantly different, however both groups were significantly different to OTC treated fish (to Cl-T; $p <0.01$, positive; $p <0.001$). The negative control group remained significantly distinct from all other groups when compared at both 11 dpi ($p<0.001$), and 21 dpi (to OTC; $p <0.05$, Cl-T; $p <0.01$, positive; $p <0.001$).

Ordinations of each sample of tank or source water (**Figure 4.4F**) were visualised throughout the trial period. Trial array tanks showed a high rate of consistency between AGD positive treatments, and shifted together between the 11 and 21 dpi timepoint. These treatments were not significantly different from one another within each timepoint, but timepoint groupings (11 dpi vs. 21 dpi) were deemed significantly disparate when analysed using PERMANOVA (F =14.2, df = 6, $p < 0.001$). The negative control tanks remained closely aligned, but were more similar to the RAS source tank where fish were smoltified, and were significantly different from all other trial treatment groups ($p< 0.05$). Filtered lab seawater, raw seawater (unfiltered, undisinfected) and CIT (amoebae inoculum water) represented more distinct communities.

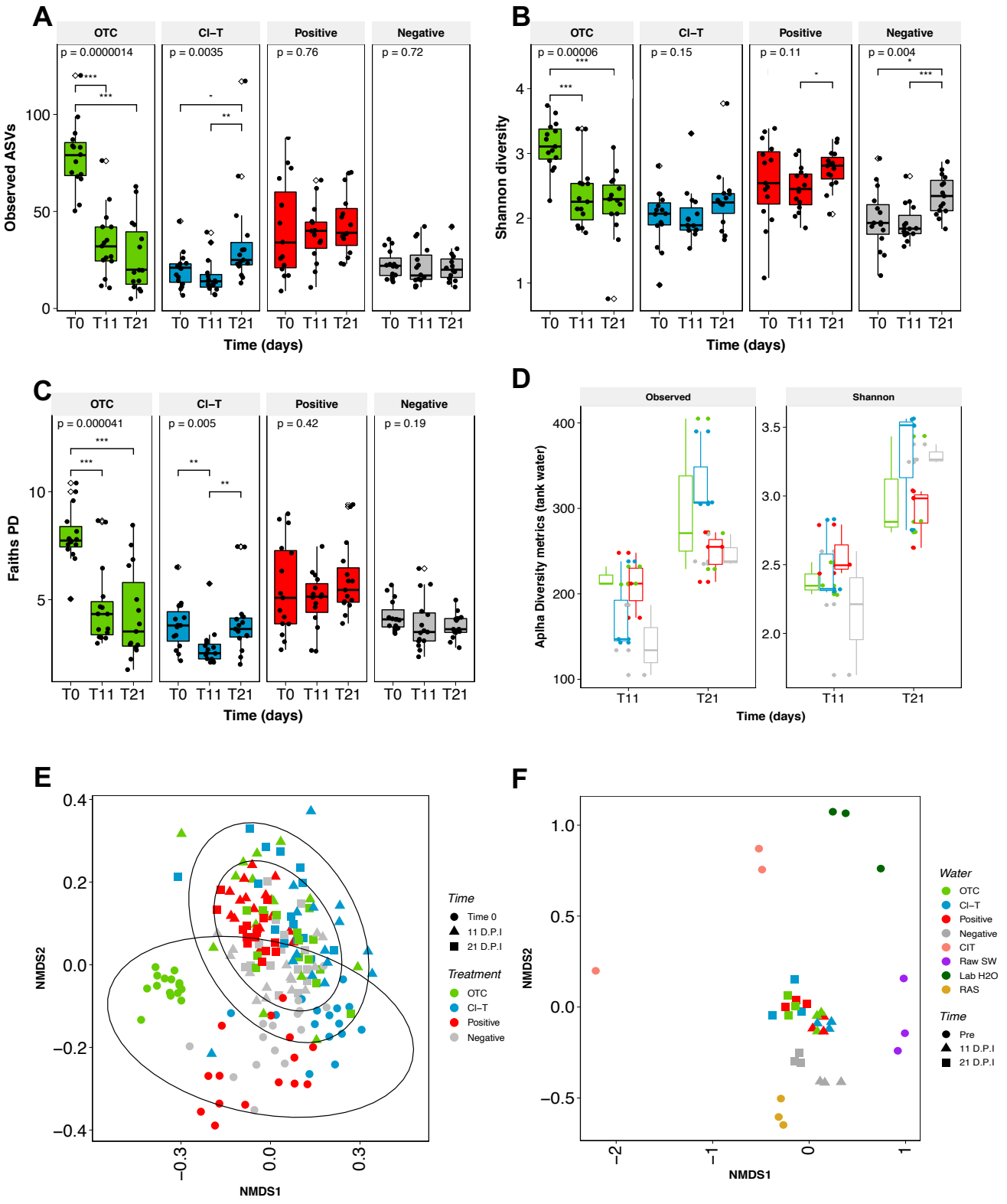

Figure 4.4. Alpha richness (A) expressed as Observed ASVs, and community diversity metrics (B,C) Shannon index and Faiths PD for gill mucus communities, along with Observed ASVs and Shannon index recorded in trial tank water (D). Black dots represent each individual sample point, unfilled diamond shape indicates outliers.p= global significance (Kruskal Wallis), pairwise significance determined by a Wilcoxon test with $p < 0.05$, $p < 0.01$ and $p < 0.001$ represented by *, **, and ***. Beta diversity visualised through Bray Curtis NMDS plots for (E) fish gill mucus and (F) holding tanks/water sources used in the trial.

Taxonomic assignment at the phylum level revealed 19 distinct taxa from fish gill mucus. The most dominant taxa in this study were *Proteobacteria* (39.01%), *Verrucomicrobia* (29.85%) and *Bacteriodetes* (16.1%) (**Figure S4.2A**). Initial timepoints for all treatment groups besides the positive control tended to have a much lower proportion of *Verrucomicrobia,* which dramatically increased post handling to 21 dpi. The OTC treatment had a much lower proportion of *Actinobacteria* comparative to other treatment groups at the initial sampling event, and subsequently a much higher proportion of *Bacteriodetes* within the 15 samples. Between all treatments the most dominant taxa were *Proteobacteria*, *Verrucomicrobia*, *Bacteriodetes* and *Actinobacteria* at 21 dpi. Tank water and experimental source water showed a strong presence of *Bacteriodetes* and *Proteobacteria*, with the two phyla composing a majority of the community with a small proportion of *Firmicutes* and *Actinobacteria* among other more cryptic taxa (**Figure S4.2B**). Source water provided to the experimental array appeared to carry a much higher proportion of *Verrucomicrobia* in contrast, with a small amount of this taxa found in both the CIT inoculum as well as the raw unfiltered seawater pumped onshore from the estuary.

The ASV assignments demonstrated 356 genera found in fish gill communities, with *Rubritalea* (33.12%), *Aquabacterium* (15.24%), *Cutibacterium* (8.83%) and *Staphylococcus* (2.95%) the most prevalent (**Figure 4.5A**). The *Rubritalea* taxon increased at each timepoint for all 4 treatment groups, after being largely absent in initially stocked fish. Gill mucus samples had high consistency between independent replicate tanks for each treatment group, but distinct treatment-based differences (PERMANOVA, $p < 0.001$). *Aquabacterium* was a dominant taxon within the gill surface mucosa in OTC, Cl-T and negative treatments, and was particularly abundant at the 11 dpi timepoint. At the 21 dpi timepoint *Aliivibrio* was prominent in the Cl-T and positive control treatments, but was not identified in the OTC group. In all AGD exposed treatments, the genus *Tenacibaculum* was present in low abundance, and increased towards 21 dpi. The exception was the Cl-T treatment at the 11 dpi timepoint, which had a much higher relative abundance of this taxa. Genus assignments for water samples (**Figure S4.3**) were characterised by *Dokdonia* (10.37%), an ASV assigned as 'uncultured' (9.68%—identified as family *Saprospiracea* and *Caldilineaceae* at family level classification), *Rubritalea* (8.36%) and *Aliivibrio* (7.85%) (**Figure S4.3**). The RAS holding tank had a small proportion of *Dokdonia*, along with the experimental tanks from all treatment groups. After having high proportions in the 11 dpi sample point, this taxon decreased by 21 dpi. In all AGD positive treatments the identified *Saprospiracea* taxa increased in prevalence at 21 dpi, along with *Aliivibrio*, which also mirrored the gill mucus samples at 21 dpi.

Differentially abundant taxa were calculated for fish gill mucus samples within each treatment group when referenced against the negative control group, with the 5 most differentially abundant taxa (*padj* < 0.01) plotted in **Figure 4.5B**. The Time 0 sample expressed higher proportions of *Dokdonia* and *Hydrogenophaga* in the OTC treatment. *Vibrio* was most prevalent at this timepoint in the negative and positive groups. The 11 dpi sample point had significant increases in *Tenacibaculum* in Cl-T and positive treatments, as well as *Aliivibrio* in the latter. At 21 dpi, *Tenacibaculum* and *Aliivibrio* were expressed at a higher proportion ($p < 0.001$) than the negative treatment. A much higher proportion of *Escherichia - Shigella* was observed in non-AGD affected fish at this timepoint.

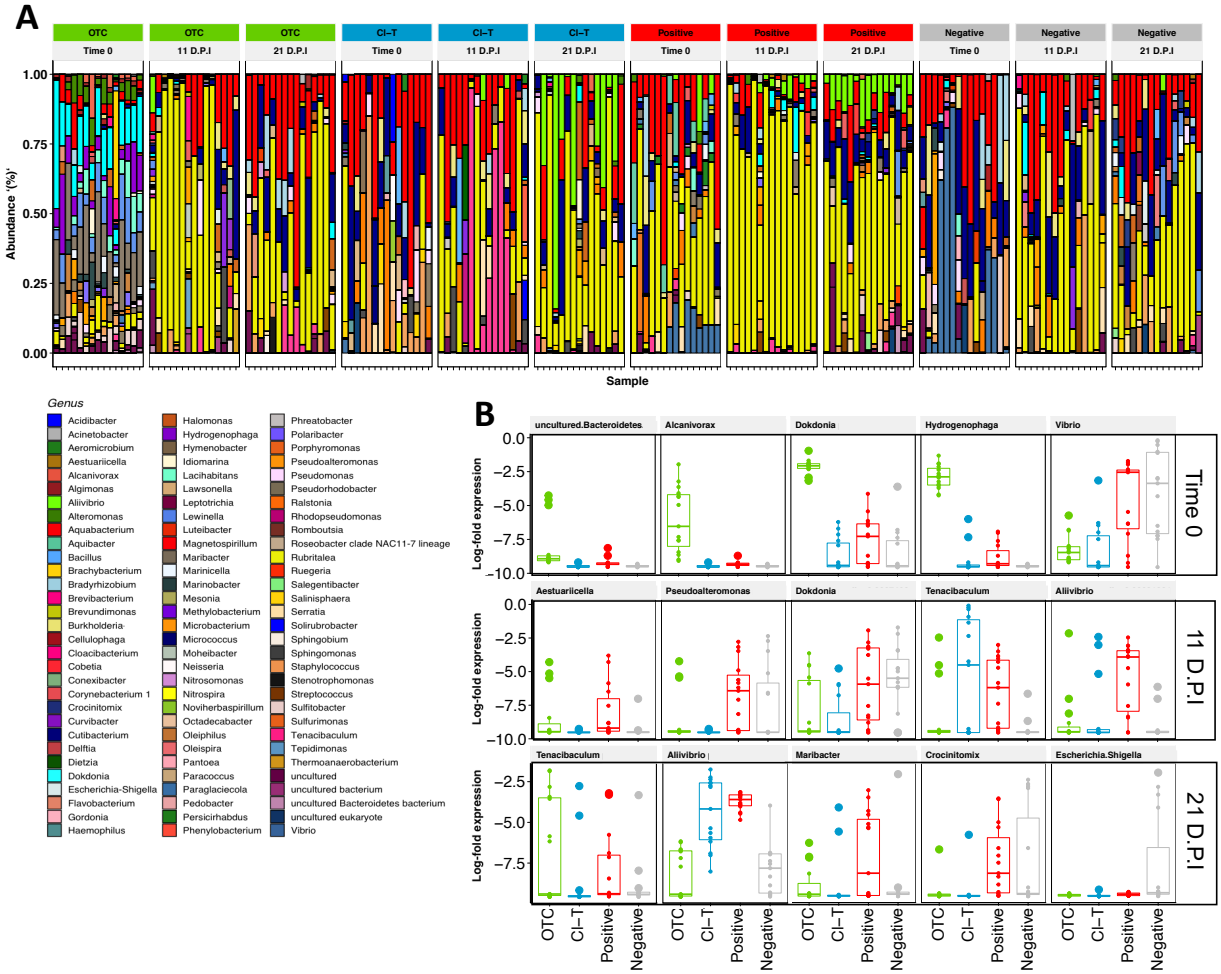

Figure 4.5. (A)-Relative abundance of the top 93 genera assigned to fish gill mucus samples at three timepoints. Each bar represents one fish gill sample for the respective treatment group. (B)-Differentially expressed (*padj* < 0.01) taxa between treatment groups within each of the three measured timepoints. Top 5 taxa are ranked based on ASV importance (ascending in significance level) and expressed as log2 fold differences. The AGD positive treatments showed higher abundance of *Aliivibrio* at 11 dpi and higher *Tenacibaculum* at both 11 dpi and 21.

During the initial inoculation process, 22 unique ASVs were identified on the gill surface of post-inoculated fish, which were common only to the CIT inoculum water and not present on the gills of unchallenged fish or in the holding tank they originated from (**Figure 4.6A**). These ASV sequences corresponded to 16 genus assignments (**Figure 4.6B**), which were dominated by *Aquabacterium*, *Aquibacter* and *Cutibacterium*. Longitudinal relative abundance of the 16 genera revealed that these three taxa along with *Allivibrio, Tenacibaculum* and *Pseudomonas* were prominently featured in all groups (**Figure 4.6C**).

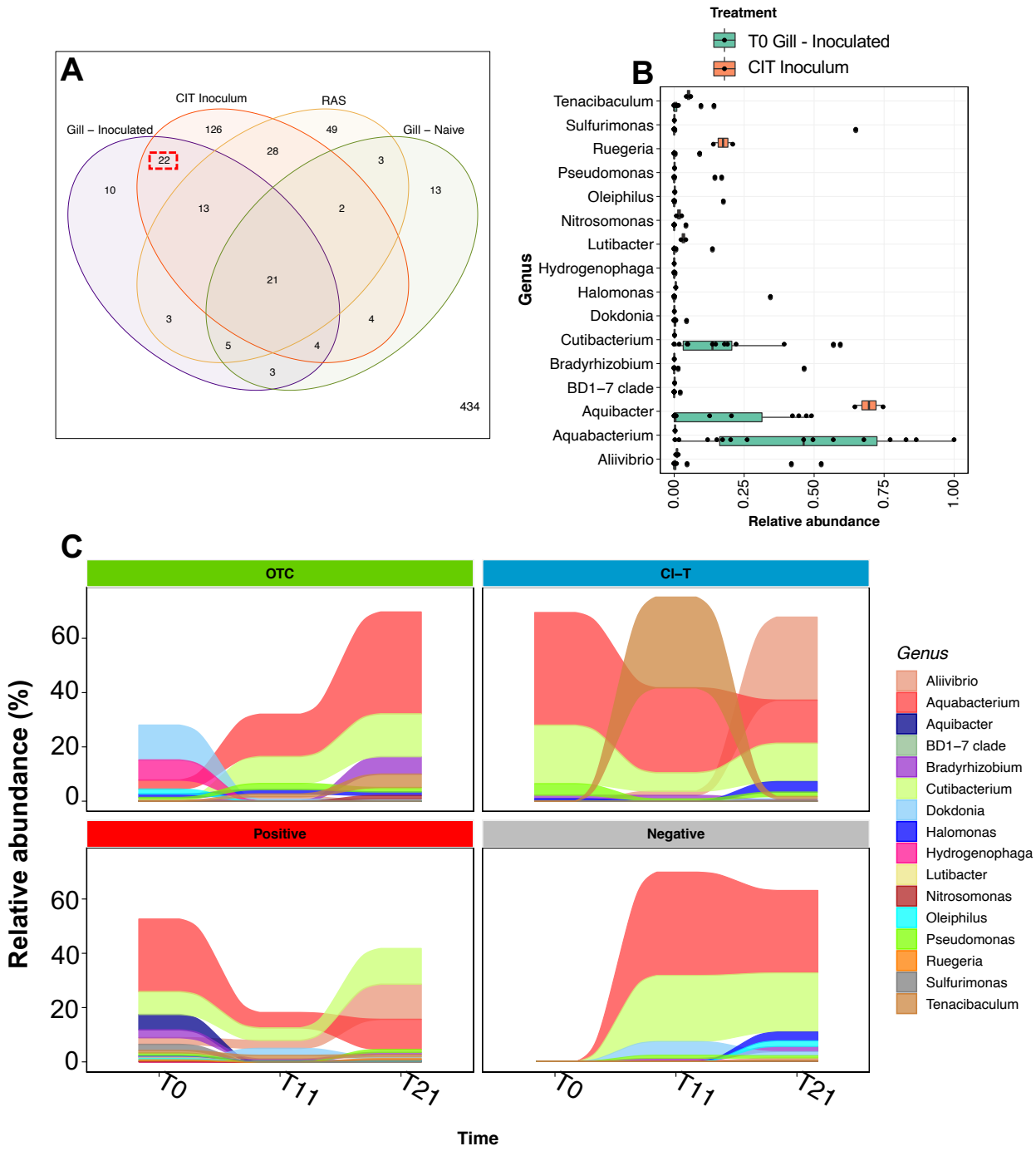

Figure 4.6. Bacterial taxa associated with AGD sources. (A) Venn diagram of shared ASV sequences between the AGD inoculum (CIT), and post inoculated gill (Time 0), naïve gill from the same cohort (Time 0) and the holding tank RAS, showing 22 ASVs shared between AGD inoculum and inoculated gills (Red dashed box). (B) Shows relative abundance of genus assigned to shared ASVs in the AGD inoculum and post challenged fish at Time 0. (C) Demonstrates the longitudinal relative abundance of representative key genus assignments over the course of the trial. Taxa including *Aliivibrio*, *Tenacibaculum*, *Cutibacterium* and *Bradyrhizobium* increase toward 21 dpi in AGD positive treatments.

4.5. Discussion

The mucosal bacterial community plays a key role in health and vitality of fish, yet we have limited understanding of the effect that commensal bacterial imbalance plays in disease susceptibility. Here, we compared the progression of AGD between groups of Atlantic salmon with modulated gill bacterial communities, and assessed the role of bacterial taxa in AGD development.

Antimicrobial treatment was effective in altering the gill mucosal bacterial community load and diversity. Colony counts from culture plates inoculated with gill mucus indicated that viable culturable bacteria numbers were reduced following antimicrobial treatment (both OTC and Cl-T). This was also reflected by disparate bacteriomic data observed after antimicrobial treatment, indicating that the gill mucus bacteria had been effectively altered. Previous studies using oxidative compounds have also demonstrated effective removal of culturable bacterial flora from the gill surface with products such as potassium permanganate (Embar-Gopinath et al., 2005b). *Gambusia affinis* immersed in the antibiotic Rifampicin also demonstrated a significant decrease in bacterial load on the skin surface (Carlson et al., 2017) lasting for 1.6 days.

After successful reduction of the bacterial load on the gills we next challenged fish with virulent *N. perurans* and compared the progression of AGD. The severity of AGD via presumptive gill score at 11 dpi (mid-point) of the trial demonstrated the gill score index was significantly higher in Cl-T comparative to the positive AGD control group. This result may indicate a more rapid progression of AGD at that stage of the challenge. In contrast, the severity of AGD in fish treated with OTC was not significantly different to untreated AGD-affected fish, despite their significant reduction in bacterial load on the gills. It is possible that initial oxidative bath treatment may have led to increased AGD susceptibility. The physiological impacts of oxidative therapeutics such as Cl-T and hydrogen peroxide on the gills include congestion of the filaments, oedema and epithelial lifting (Adams et al., 2012; Kiemer and Black, 1997; Powell et al., 2015; Powell and Perry, 1999). Denuding the mucosal bound layer may hinder any innate protective functions. While it was demonstrated that mechanical damage to the gill epithelium did not lead to increased AGD progression (Adams et al., 2009), limited data exists regarding pre-exposure to

oxidative chemicals prior to *N. perurans* challenge. It is possible the downstream impact of stress from such oxidative treatment may compromise the innate immunity of the fish, facilitating favourable colonisation of *N. perurans* and faster progression of AGD. Chloramine-t has been identified as a non-specific immune suppressant in rainbow trout, where fish exposed to 5 ppm immersion for 3 h demonstrated a decrease in plasma lysozyme and serum bactericidal activity (Yavuzcan Yildiz et al., 2009). Other oxidative topical treatments such as hydrogen peroxide and peracetic acid also induce both physiological and oxidative stress on Atlantic salmon (Soleng et al., 2019; Vera and Migaud, 2016).

Bacterial count data at 11 dpi showed the gills of fish bathed in Cl-T were rapidly recolonised with bacteria, although the OTC group remained at negligible levels. Taxonomically, genus level bacteria between treated and untreated groups were vastly different, indicating that antimicrobial action had led to a community level imbalance. It is likely that the bacterial taxa susceptible to OTC and Cl-T may have been removed, and subsequently replaced on the gill surface with compound-resistant species which were not able to be cultured and thus not able to be quantified using the count plate method. We observed high levels of *Tenacibaculum* in the Cl-T group at the 11 dpi (**Figure 4.6**). Similarly, (Genaro Sanchez et al., 1996) observed detrimental impacts post Cl-T treatment in rainbow trout, where the skin microbial layer was left infection prone and colonised by secondary opportunists (*Tenacibaculum* and *Pseudomonas).* It remains possible that the increased incidence of known pathogenic bacteria such as *Tenacibaculum* in the Cl-T group may have also contributed to any increase in the onset of AGD noted in this study (**Figure 4.2**). Several publications have examined the incidental co-abundance of *Tenacibaculum* and amoebic branchialitis, and surmised that severity may increase in the presence of both pathogens (Downes et al., 2015; Powell et al., 2005b; Slinger et al., 2020a). Evidence for bacterial co-factor virulence in other ectoparasitic conditions exist, including Ich (*Ichthyophthirius multifiliis*) in the presence of *Aeromonas hydrophila* (Liu and Lu, 2004; Xu et al., 2012), and fish lice (*Argulus coregoni*) when co-infected with *F. columnare* (Bandilla et al., 2006).

Progression of AGD at the 21 dpi sampling point was verified by an increase in mean gill score and increased *N. perurans* 18S gene abundance. These were highest in Cl-T treated fish, and significantly different to the untreated positive control group. Concomitantly, branchial histopathology of fish challenged with *N. perurans* indicated that the Cl-T bathed group also had the highest percentage of lesion affected filaments, differing significantly to the AGD positive control. Taken together, results of presumptive gill scoring, parasite qPCR assays, and histopathology suggested that AGD had advanced marginally further within the group of fish treated with Cl-T prior to challenge. In similar scenarios, potassium permanganate ($KMnO_4$)

treatment of channel catfish (*Ictalurus puntactus*) caused bacterial diversity imbalances of the skin, which was further exacerbated when challenged with *Flavobacterium columnare* (Mohammed and Arias, 2015). Contrastingly, fish not treated with $KMnO_4$ (pre-challenge) retained a higher bacterial diversity post infection. Other studies have postulated that fish may be more susceptible to secondary pathogens as a result of a prior dysbiosis (Reid et al., 2017; Schmidt et al., 2016b). Further research investigating the mechanisms of such disease susceptibility is required to improve our understanding of how bacterial dysbiosis affects disease pathogenesis.

Viable bacterial counts increased in all experimental groups (besides OTC) longitudinally with the Cl-T, positive and negative control groups finishing 21 dpi at similar $CFU.mL^{-1}$ loads. The increasing bacterial load in both untreated AGD challenged and AGD naive fish may point to the tank environment taking some time to establish (after transition from RAS to flow-through water sources). Bacterial numbers in aquaculture tank systems have been previously observed to increase 40-fold over 24 days post establishment (Rojas-Tirado et al., 2017). The culturable bacterial load of healthy rainbow trout (*Oncorhynchus mykiss*) gills was reported to be around 4.95×10^3 $CFU.mL^{-1}$ (Ostland et al., 1990). These data were obtained from gill material of 7 holobranchs and appear to agree with the current study compared to a single hemibranch surface. Bacterial sequence data was largely in agreement with aerobic count plate data. Observed ASVs and Shannon diversity for the Cl-T, positive and negative fish peaked at the 21 dpi sample point, possibly indicating that the gill mucosa was accumulating bacterial taxa through the trial duration. These data are supported by the alpha diversity values recorded for tank water, where diversity and richness increased from 11 dpi to 21 dpi. In contrast, diversity and richness of gill mucus in the OTC treated group decreased over time. Reduction in commensal bacterial diversity and richness post-antibiotic treatment has been well documented in a range of aquatic species. For example, Atlantic salmon (Navarrete et al., 2008) when treated with in-feed OTC showed a dramatic reduction in diversity of intestinal bacteria. Both (Rosado et al., 2019) and (Legrand et al., 2020) demonstrated that the microbial perturbation of such antibiotic usage can last upwards of 18 days. In this study we did not observe a significant dysbiosis over the progression of AGD at 21 dpi, compared to other studies which have observed such perturbations in health-affected fish (Legrand et al., 2018; Reid et al., 2017). However, the infection load of *N. perurans* and consequential development of gill lesions in the current study suggested a light to moderate disease response. Previously we had demonstrated that more advanced AGD contributes to a lower mucus bacteria diversity (Slinger et al., 2020b, 2020a). Interestingly, the presence of both *Winogradskyella* and *Staphlococcus* in water samples from AGD affected tanks agree with previous work (Embar-Gopinath et al., 2005a). This study cultured

colonies from gill mucus swabs to obtain several bacterial isolates which may have been present in concert with AGD affected Atlantic salmon, but absent in fish naïve to the condition. Historically, numerous challenge methodologies have been employed for inducing AGD experimentally, including cohabitation of trojan AGD affected fish to a naïve cohort (Roberts and Powell, 2005b; Zilberg and Munday, 2000), or by excising the gill basket of AGD affected individuals to harvest gill-attached trophozoites (wild-type) (Adams et al., 2009; Morrison et al., 2004; Pennacchi et al., 2014). The current study employed an immersion challenge using water from a dedicated AGD constant infection tank (CIT), which passaged AGD affected hosts to maintain *N. perurans* load, as per previous studies in this facility (Taylor et al., 2021a; Wynne et al., 2020a). Due to difficulty or impracticality cultivating axenic *N. perurans* for fish challenge (Kent et al., 1988; Morrison et al., 2005; Vincent et al., 2007), all *in vitro* and *in vivo* methods appear to be concomitantly linked with bacterial growth. It is logical to assume that because of the xenic nature of *N. perurans* exposure in all inoculation scenarios, bacterial components are also simultaneously exposed to the gill. This study characterised the bacterial biomass associated to the immersion challenge inoculum, which may be linked to the ecology of *N. perurans*. There were 22 distinct ASVs identified that colonised salmon gill surfaces immediately following inoculation of the tanks with water containing *N. perurans*. The 17 genus level assignments were prominently featured on the gills in most groups. The bacterial taxa which were significantly differentially expressed in AGD positive groups to 21 dpi included known pathogenic taxa, *Aliivibrio*, *Pseudomonas* and *Tenacibaculum*. These specific genera have been identified in high abundances in several fish disease settings including gut enteritis of yellowtail kingfish (*Seriola lalandi*), as well as winter ulcer disease and sea louse infestations in Atlantic salmon (Karlsen et al., 2017; Legrand et al., 2020; Llewellyn et al., 2017). The limitation of having bacterial rich amoebae for AGD research means that to comprehensively examine the impact of bacterial co-factors an experimental protocol incorporating axenic trophozoites would need to be completed. A vaguely assigned ASV (uncultured) at genus level made up a large component of the tank (water) community in all AGD positive groups at the latter stages of the trial. This classification was investigated via family level assignments to be predominately composed mainly of *Saprospiracea* and to lesser extent *Caldilineaceae*, identified using the NCBI BLAST tool (Seq. ID AB625329.1 and JF514230.1). Although the exact species is unknown the groups of *Saprospiracea* related taxa are known for hydrolysis and utilisation of complex carbon sources (McIlroy and Nielsen, 2014).

In conclusion the present study suggests that reducing gill bacteria that were sensitive to orally administered OTC did not significantly affect the progression of experimentally induced AGD.

However, bath treatment with chloramine-t prior to amoebic challenge led to marginal advancement of AGD in salmon smolt. We demonstrate that AGD developed with different levels of bacterial dysbiosis, and progressed concurrently with increased colonisation of potential secondary pathogenic bacterial taxa including *Aliivibrio* and *Tenacibaculum*, coinciding with the peak of the AGD severity observed in this study. To examine this further, the functional role or relationship of these bacterial taxa in AGD development warrants further enquiry.

4.6. Acknowledgements

We gratefully thank CSIRO and Tassal Group Ltd. for supporting this study. This research was enabled by a PhD scholarship awarded to JS from the Institute for Marine and Antarctic Studies (University of Tasmania). The authors wish to acknowledge Tansyn Noble for review of this manuscript, and to Lambertus Koster for contribution to husbandry of salmon stock used in this study.

Chapter 5. Bacteriomic profiling of branchial lesions induced by challenge with *Neoparamoeba perurans* reveals commensal dysbiosis and an association with *Tenacibaculum dicentrarchi* in AGD-affected Atlantic salmon (*Salmo salar* L.)

This chapter is a verbatim reproduction from the following published paper:
Slinger, J., Adams, M.B., Wynne, J.W., 2020. Bacteriomic Profiling of Branchial Lesions Induced by *Neoparamoeba perurans* Challenge Reveals Commensal Dysbiosis and an Association with *Tenacibaculum dicentrarchi* in AGD-affected Atlantic salmon (*Salmo salar* L.). Microorganisms 8, 1–17. https://doi.org/10.3390/microorganisms8081189

5.1. Abstract

Amoebic gill disease is a parasitic condition that commonly affects marine farmed Atlantic salmon. The causative agent, *Neoparamoeba perurans*, causes significant and well characterised host response within the gill. The effect that AGD-induced host response has on the commensal microbial community of the gill has not been described. A 16S rRNA sequencing approach was employed to profile changes in bacterial community composition within AGD-affected and non-affected gill tissue. The bacterial diversity of biopsies taken from lesion and (proximal) non-lesion tissue was significantly lower in the AGD-affected fish compared to AGD naïve controls. Furthermore, within the AGD-affected tissue, lesions appeared to contain a significantly higher abundance of the *Flavobacteria*, *Tenacibaculum dicentrarchi* compared to the non-lesion tissue. Quantitative PCR specific to both *N. perurans* and *T. dicentrarchi* was used to further examine the co-abundance of these known fish pathogens. Pearson correlation analysis of log transformed copy numbers indicated a moderate positive association between these organisms. Taken together, the present study sheds new light on the complex interaction between the host, parasite and bacterial communities during AGD progression. The role that *T. dicentrarchi* may play in this complex relationship requires further investigation.

5.2. Introduction

The outer gill surface of teleost fish represents a unique and dynamic landscape where microbial antigens within the external milieu attempt to invade the mucosal interface, whilst the host immune system attempts to overcome these continuous insults (Cabillon and Lazado, 2019; Llewellyn et al., 2014). Furthermore, collateral damage during this conflict can lead to profound changes in the commensal microbial community, which may ultimately contribute to complex disease pathologies. Previous research has shown that the seawater environment contains up to 10^7 organisms per millilitre (Whitman et al., 1998), and therefore, represents a rich source of microbes that, under certain conditions, can have negative effects on the host. A delicate balance exists between commensal and opportunistic pathogens, however under certain conditions such as disease or poor environmental conditions this microbial balance can be lost, leading to a dysbiosis where opportunistic species dominate (Derome et al., 2016).

Several examples exist where the commensal host-associated microbiota is significantly impacted as a result of opportunistic pathogen infection. The commensal microbiomes of Asian seabass (*Lates calcarifer*) and largemouth bronze gudgeon (*Coreius guichenoti*) demonstrated an appreciable loss of richness and diversity when affected by tenacibaculosis and furunculosis respectively (Li et al., 2016; Miyake et al., 2018). Similarly, the microbiome of salmon skin was observed to be in a state of imbalance at the interface of the pathogen and host during infection with ecoparasitic sea lice *(Lepeophtheirus salmonis*) (Llewellyn et al., 2017). Regular aquaculture operations can also influence bacterial communities, with soy-based dietary additives (Reveco et al., 2014) and husbandry practices such as seawater transfer and handling (Lokesh and Kiron, 2016; Minniti et al., 2017) having significant impacts on the internal and external microbiota in respective *in vivo* studies with Atlantic salmon. Commensal probiotic treatments can provide some microbial resilience to finfish, as observed in both black molly (*Poecilia sphenops*) and Arctic char (*Salvelinus alpinus*), when a probiotic additive was seen to lessen the detrimental impact of two significant pathogenic bacterial species, *Vibrio anguillarum* and *Flavobacterium psychrophilum* respectively (Boutin et al., 2013; Schmidt et al., 2017).

The gills of fish are a structurally and functionally complex organ that need to respond rapidly to adverse circumstances. Amoebic gill disease (AGD) is a parasitic gill condition affecting Atlantic salmon (*Salmo salar*) aquaculture globally. The aetiological agent of the condition is *Neoparamoeba perurans* (Crosbie et al., 2012), a marine free-living amphizoic amoeba, which upon attachment to the gill surface elicits clinical responses including grossly visible white mucoid patches upon the gills, respiratory distress, hypernatremia, inappetence and mortality if untreated (Munday, 1986; Zilberg and Munday, 2000). Multifocal gill lesions are dominated by hyperplasia of the

respiratory mucosa with variable inflammatory infiltration (Bridle et al., 2006; Marcos-López and Rodger, 2020; Morrison et al., 2007, 2006b; Nowak et al., 2014; Pennacchi et al., 2016; Wynne et al., 2008). While the host response to AGD is well characterised, the impact (and interactions) that AGD may have with the commensal bacterial community is largely unknown to date. Previous studies have investigated the relationship between specific candidate bacteria and AGD development. A culture independent assessment of AGD affected farm salmon demonstrated a possible link between amoebic infection and *Psychroserpens* taxa (Bowman and Nowak, 2004). Similarly, experimentally induced AGD lesion incidence was exacerbated in the presence of *Winogradskyella* sp. in Atlantic salmon (Embar-Gopinath et al., 2005a).

It may be possible that *N. perurans* directly interacts with the microbial population on the gill, either by eliciting localised agents responsible for lesion formation, or by fulfilling a role as a potential vector to harbour and transport bacterial taxa to the gill surface. Indeed, complex and often symbiotic relationships exist between amoebae and bacteria, that may ultimately effect pathogeneses in the host. *In vitro* studies have shown that *N. perurans* produces cytolytic extracellular products (ECPs) (Bridle et al., 2015; Cano et al., 2019) which are likely to underly necrotic fenestrations observed at amoebae attachment sites upon gill lesions of AGD affected fish (Butler and Nowak, 2004; Roubal et al., 1989; Wiik-Nielsen et al., 2016). Pathogenic amoebae such as *Entamoeba histolytica* also utilise the production of proteases to enhance host cellular degradation once attached to the tissue surface (Mirelman, 1987). Further to this, it has been shown that these processes have enhanced disease severity when Entamoebae trophozoites utilise known pathogenic bacteria as a feed source (Galván-Moroyoqui et al., 2008) that augment cytolytic effects. The attachment mechanism of *N. perurans* to gill surfaces may also be bacteria-assisted, as with other pathogenic amoebae species such as *Acanthamoeba* (causative agent of eye keratitis) favouring attachment sites in the presence of key bacteria which are known to produce specific cytopathic proteases (Neelam and Niederkorn, 2017).

Techniques in culture independent bacterial profiling now exist to give greater clarity and understanding of how commensal host microbiota on the gill surface interface responds to amoebic (*N. perurans*) insult. Here, we hypothesise that changes in branchial surface morphology and cellularity at lesion sites associated with infection by *N. perurans* may alter the bacteriomic profile of infected gills in Atlantic salmon. The aims of this study were therefore to investigate whether the diversity and richness of branchial microbial communities could be altered by experimentally induced AGD, investigate whether these indices vary discretely between diseased and non-diseased gill tissues and ascertain whether particular bacterial taxa are prominent in AGD-affected fish *in vivo*.

5.3. Materials and Methods

All animal procedures were approved under application (#2018-9) by the Queensland CSIRO Animal Ethics Committee under the guidelines of the Australian Code of Practice. Fish used for this work were humanely killed (immersion bath of 100 ppm AQUI-S anaesthetic) prior to sampling.

5.3.1. Amoebic Challenge and 16S rRNA Bacterial Community Analysis

Experimental Challenge with Neoparamoeba perurans

Following habituation (7 d) in seawater, 50 Atlantic salmon smolt (naïve to AGD) were transferred to a 1000 L seawater (35 ppt) flow-through tank with temperature maintained at 15±0.5°C, pH at 7.8±0.1 and dissolved oxygen maintained between 90-100% saturation. Fish were fed daily to satiation using a commercial 3 mm pellet (Nutra, Skretting P/L, Australia). The remaining 500 fish were purposed for use in an AGD challenge trial (Wynne et al., 2020a), and were exposed to wild-type gill associated trophozoites of *N. perurans* as follows. A sufficient volume of water (taken from a recirculating system containing AGD affected Atlantic salmon) was introduced to the holding system in sufficient volume to achieve a final concentration of 100 *N. perurans*.L^{-1} (1 h static exposure). The concentration of infective amoebae was determined from 1 L samples of system water centrifuged at 4000 *g* to concentrate cells into a final volume of 10 ml. Wild-type *N. perurans* stock concentration was then enumerated by averaging repeated trophozoite counts (n=10) on a haemocytometer. A subset of 33 Atlantic salmon were taken from the exposure tank and stocked into a single 500 L flow-through seawater tank. Fish were subsequently maintained at 15±0.5°C, pH 7.8±0.1, 35 ppt and dissolved oxygen saturation between 90-100%. The infected fish were fed daily to satiation with a commercial diet (Nutra 3 mm, Skretting P/L, Australia) until sampling at 21 days post-infection (dpi).

Tissue Biopsy Sampling

For AGD affected fish (n=10), the dorsal region of the third holobranch on the right-hand side of the gill basket (RH3) was excised and examined for viable AGD lesions (**Figure 5.1**). A gill filament biopsy of up to 3 hyperplastic AGD lesions was excised a using sterile one-use biopsy punch (2 mm diameter) methodology adapted from prescribed methods in (Pennacchi et al., 2016, 2014), and placed into a 1.5 ml tube containing 1 ml RNAlater solution. An area adjacent to each lesion of unaffected (normal) gill filaments were then excised using a new punch and placed into a separate 1.5 ml tube containing 1 ml RNAlater solution. RNALater preserved samples were stored in the freezer at -20°C until DNA extraction.

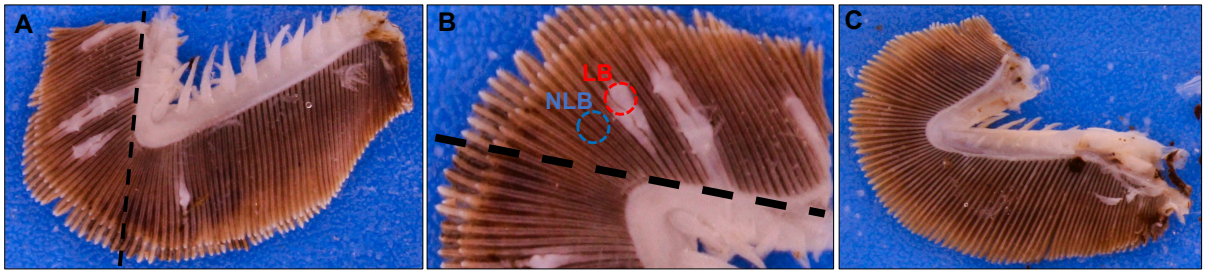

Figure 5.1. (A) - Example of AGD affected RH3 hemibranch, and (B) – subset of (A), showing biopsy punch samples obtained from AGD originating gross gill lesions (red) and adjacent unaffected tissue (blue) located in the dorsal region (above dotted black line). (C) – depicts hemibranch from AGD naïve smolt, with no visible gross pathology

Gill Mucosal Swabs

The anterior surface of the third right hemibranch (LH3) in fish naïve to AGD was swabbed by holding open the operculum and gently rotating (x3) a disposable sterile cotton swab over the entire length of the hemibranch. The swab handle was then trimmed using sterilised dissection scissors and placed into a 1.5 ml tube containing RNAlater (1 ml) and stored at -20°C until DNA extraction.

Gill Histology

Gill arches were collected and processed for histological interpretation from AGD affected salmon as detailed by Wynne et al. (2020). Briefly, gill arch tissues were fixed in seawater Davidson's solution for 24 hrs before transfer to 70% ethanol prior to processing. The gill arch was dehydrated and infiltrated with wax, prior to embedding and sectioning on a microtome at 5 µm. Sections were de-waxed and stained with haematoxylin and eosin and a subset of AGD positive and AGD naïve samples (n=9) were observed under a light microscope (Olympus, Hamburg, Germany) and photographed (Nikon DS-Ri2, Nikon Instruments, Tokyo, Japan).

Water Sample Collection

A sample of the culture water was collected via filtration of pooled replicates (700 ml x 3) to make a total of ~2 L^{-1} of tank water. This was completed using a peristaltic pump (RP-100 series, Lachat Instruments, Wisconsin) that passaged water across a 0.22 µm Sterivex™ (Millipore) filter membrane to retain bacterial cells. The Sterivex™ filter chamber was then flooded with 2 ml of RNAlater solution to fix bacterial cells and then stored at -20 °C prior to DNA extraction.

5.3.2. DNA Extraction and Purification

All samples underwent DNA extraction using the DNeasy Blood and Tissue Kit as per manufacturer's protocol for animal tissue samples (Qiagen, Hilden, Germany). Samples were lysed (5 h), before washing and elution were completed. For both swab and tissue samples, total genomic DNA was assessed for yield and quality using a Nanodrop ND-1000

spectrophotometer (Life Technologies, Carlsbad, US). Samples were stored at -80°C until sequencing.

5.3.3. 16S Amplicon Sequencing

Sequencing was performed at the University of New South Wales, Ramaciotti Center for Genomics (Sydney, Australia) via an Illumina Miseq platform with 300 base pair (bp) paired end reads. The sequencing targeted the V1-V3 hypervariable region of the 16S rRNA gene in this study ("27F"AGRGTTTGATCMTGGCTCAG; and "519R"GWATTACCGCGGCKGCTG) as per the protocols in (Lane, 1991; Lane et al., 1985). A total of 33 cycles were performed with normalised DNA at an average of 10 $ng.\mu l^{-1}$. Samples in this study were composed of two separate sequencing runs, with both runs including a mock positive control (ZymoBIOMICS Microbial Community Standard, Zymo Research), and two negative controls (blank swab process control and blank DNA extraction laboratory control).

5.3.4. Bioinformatic and Statistical Analyses

Raw Illumina amplicon sequencing data files were processed using the open-source software pipeline "Quantitative Insights into Microbial Ecology 2"QIIME2 (Caporaso et al., 2010). Paired end sequences from the forward and reverse reads were merged for each sample and were denoised using the q2-dada2 plugin (Callahan et al., 2016) with default parameters. Quality control including chimeric sequence removal from the dataset was completed during dada2 processing, along with subsequent removal of host DNA and exclusion of chloroplast and mitochondrial sequences. Amplicon Sequence Variants (ASV's) were classified taxonomically using the classify-sklearn method in the QIIME2 q2-feature-classifier plugin using default parameters (Bokulich et al., 2018). The SILVA 16S rRNA 99% taxonomy database release 132, (Quast et al., 2012), was used as reference sequences for taxonomic classification.

5.3.5. Statistical Analysis

All statistics were performed in R version 3.6.0 (R Core Team, 2019). Samples were rarefied using R package QsRutils (Quensen, 2020). Using the Phyloseq package (McMurdie and Holmes, 2013) taxonomic assignments were generated and alpha diversity indices calculated (Observed ASV's, Shannon diversity, Faith's phylogenetic distance). The alpha diversity metrics were analysed via non-parametric means (Kruskal-Wallis test) and further pairwise comparisons using a Wilcoxon Test (Rank Sum Test). Beta-diversity comparisons were made via NMDS using Bray Curtis pairwise distances. Differences between groups was analysed using the Vegan package ANOSIM (Oksanen et al., 2018). Differential abundance testing was completed using the DeSeq2 package found in (Love et al., 2014). All figures were produced using the R package ggplot2 (Wickham, 2016).

5.3.6. Quantitative PCR

PCR assays were used to quantify the abundance of *T. dicentrarchi* and *N. perurans* in the 10 LB and 10 NLB samples from AGD affected fish. A TaqMan quantitative PCR was designed to amplify a 153 bp region of the *T. dicentrarchi* 16s rRNA gene. Specific primers and probe were designed based on a multiple sequence alignment of 16S *Tenacibaculum* spp. **Figure S5.1** and are shown in **Table 5.1**. PCR was performed in a single-plex 25 µl reaction containing 2X buffer, 50 mM MgCl, 10mM dNTP, 10 uM forward and reverse primer, 10 uM of probe and 0.4 units of DNA Taq polymerase (Sensifast, Bioline). Each reaction contained 2 µl of normalised template DNA (30 $ng.ul^{-1}$). PCR reactions were subjected to the following thermal cycling: 95°C for 10 min, then 95°C for 15 s and 60°C for 1 min for 40 cycles, and a hold of 4°C. A quantitative PCR assay of *N. perurans* was also performed using methods previously described by (Downes et al., 2015). Quantitative PCR data was analysed as a relative standard curve, which was produced using a cloned plasmid for each amplicon **Figure S5.2**. Plasmid DNA was cloned using the pGEM-T easy vector system as described previously (English et al., 2019).

Table 5.1. Nucleotide sequences of primers and probes both designed and used in this study, for the real time PCR detection of *T. dicentrarchi* and *N. perurans* DNA fragments

Assay (gene)	Primer	Sequence (5'-3')	Length	Ref
T. dicentrarchi	FWD	TAACATTATGCTTGCATAGATGACGA	26 bp	*Current study*
(16S rRNA)	REV	AGCCTTATGATAATTTGTAAATACCCATG	29 bp	
	Probe	FAM-CCTTTAGAAATGAAGATTAATACTCCATAATGTAGTGATTCGG-MGB	43 bp	
N. perurans	FWD	AAAAGACCATGCGATTCGTAAAGT	24 bp	(Downes et
(18S rRNA)	REV	CATTCTTTTCGGAGAGTGGAAATT	24 bp	al., 2015)
	Probe	FAM-ATCATGATTCACCATATGTT-MGB	20 bp	

5.4. Results

5.4.1. AGD Pathology

Gross clinical signs of AGD including raised multifocal lesions on the gill surface were observed in AGD affected fish (**Figure 5.1**). Macroscopic gill lesions were clearly distinguishable and were successfully biopsied from AGD affected fish. Collectively these fish had an average gross gill index of 3.30. Gill lesions were not observed in salmon unexposed to *N. perurans*. Histologically, AGD affected fish displayed multifocal lamellar hyperplasia, lamellae fusion, interlamellar vesical formation and oedema (**Figure 5.2**) in close association with trophozoites of *N. perurans*.

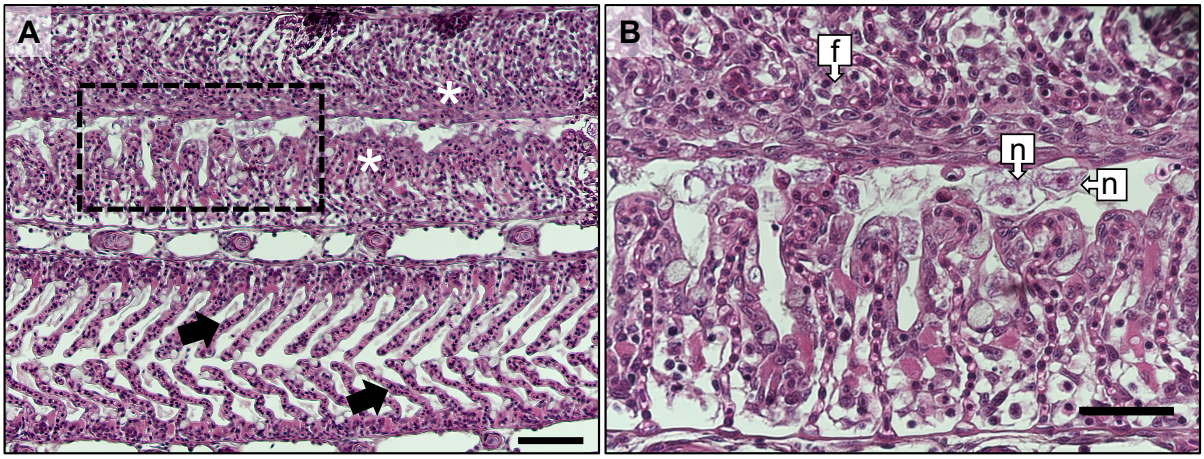

Figure 5.2. A – Gill filaments from AGD affected fish, showing anatomically normal secondary lamellae in lower filaments (black arrows), adjacent to a hyperplasic lesion induced by *N. perurans* (white asterisks), Bar = 100 μm. Inset border (dashed line box) corresponds to (B), at 40x magnification. B – frame subset of (A) showing fusion of secondary lamellae (f), and *N. perurans* trophozoites (n) with nucleus and endosymbiont present (Bar = 100 μm).

5.4.2. 16s Amplicon Sequencing

The V1-3 region of the 16S rRNA gene was successfully amplified from all DNA samples, and sequenced as 300 bp paired end read. For subsequent microbiome analysis the read depth was subsetted to 4,100 reads to provide even sampling effort. All sequence reads were deposited into the SRA under BioProject SUB7661026.

Alpha and Beta Diversity

Assessment of bacterial taxa richness and diversity was used to compare community structure of AGD unaffected (naïve), Lesion biopsy (LB) and Non-lesion Biopsy (NLB) smolt groups. There was a significant difference in observed ASV's, between sample groups (KW test, X^2=6.56, p<0.05; **Figure 5.3A**), with naïve fish having higher species richness than both AGD sample groups (Wilcox; p<0.05). For population diversity metrics, the Shannon species evenness (KW test, X^2=17.21, p<0.001; **Figure 5.3B**) was significantly different between two groups, specifically the Naïve group showed higher species evenness than both LB (Wilcox; p<0.001) and NLB samples (Wilcox; p<0.01), and the naïve vs both AGD sample groups (Wilcox; p<0.001). Simpson diversity (KW test, X^2=13.44, p<0.01; **Figure 5.3C**) was also highest in the naïve group, which was not different to NLB, but significantly higher than LB (Wilcox; p<0.001). The NLB group also proved to be significantly different to the LB group (Wilcox; p<0.05). Overall these results point to a more abundant and diverse bacterial community present within the naïve samples and a decreasing bacterial diversity within the AGD lesion site.

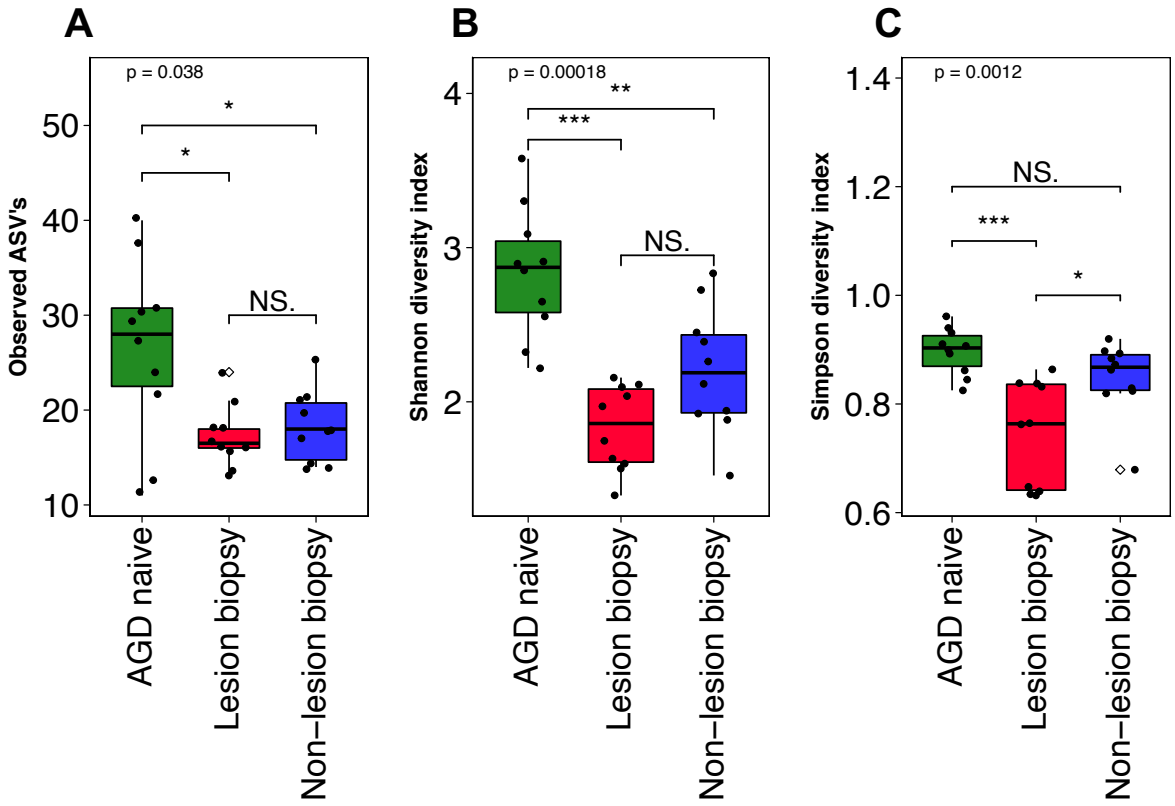

Figure 5.3. Measures of bacterial alpha diversity measures in naïve and AGD affected salmon. Mean species richness (A) was higher in naïve smolt than both AGD groups, whilst diversity (B and C) were highest in naïve fish, and significantly different between naive and LB AGD groups. Black dots represent each individual sample point, unfilled diamond shape indicates outliers. P = global significance (Kruskall Wallis), pairwise significance determined by a Wilcoxon test with $p<0.05$, $p<0.01$ and $p<0.001$ represented by *, **, and ***.

Comparison of between-sample variance of bacterial communities by group was investigated by non-metric multidimensional scaling plots using ranked distance metrics (**Figure 5.4A**). Statistical analysis of similarity (ANOSIM) between lesion biopsy groups, demonstrated a significant difference between bacterial community composition of naïve, LB and NLB groups (ANOSIM, $p<0.05$). Between AGD sample comparison (**Figure 5.4B**) indicates some overlap and variation between the LB and NLB groups, where there was appreciable interspecific (fish-to-fish) variation. (**Figure 5.4B**).

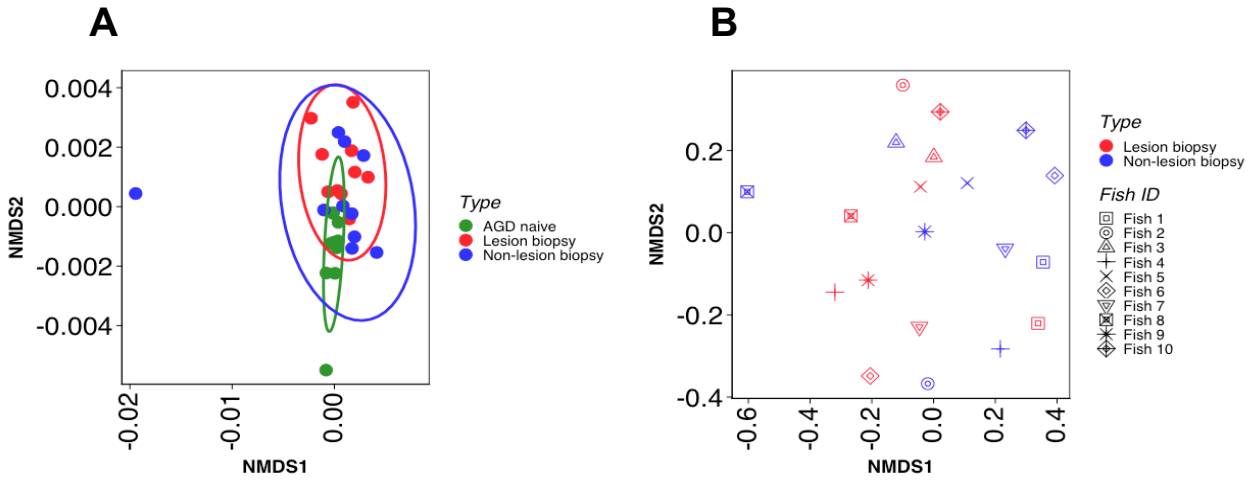

Figure 5.4. Beta diversity analysis using non-metric multidimensional scaling of Gower distance dissimilarity of (A), all sample types (stress = 0.17) and Bray Curtis ranked distance (B), LB and NLB samples denoting individual fish by shape (stress = 0.21). These data indicate that groupings between sample type were distinct, and that fish-to-fish variation of the LB and NLB groups was high.

Taxonomic Assignment and Composition

A total of 4856 ASVs were identified across our samples and were assigned to 38 phyla, 202 order, 299 classes and 471 genera. Differences in taxa abundance was observed between the US, LB and NLB samples. At the phylum level, there was a shift in dominant phyla with an increase in Bacteriodetes abundance (37.2%), which was increased from Naïve (11.3%) and NLB (6.3%) sample groups (**Figure 5.5A**). The predominant phylum for Naïve and NLB remained Proteobacteria (68.1% & 81.6%), which was reduced in LB samples (49.6%) (**Figure 5.5A**). Actinobacteria was consistent across all three groups, but highest in LB samples (12.2%). Several phyla were present at <1% abundance throughout. At the genus level, considerable diversity was observed across the naïve samples, with a higher number of low abundance assignments, the profile of which aligned closely to the source water sample at that timepoint (**Figure 5.5B**). The NLB sample group had a lower number of genus taxa than the AGD naïve group, but was more diverse than the LB samples. In the LB samples we observed a high mean abundance (70.7%) of a single ASV classified to the genus *Tenacibaculum* in five of the ten samples, and lower abundance in all other LB samples (**Figure 5.5B**). In contrast this ASV was significantly less abundant or absent in the naïve and NLB samples, specifically being absent in four of the ten NLB samples, with a mean abundance of 0.83% in remaining samples and only one sample >1%. Closer inspection of the taxonomic classification of this ASV using a global alignment tool, Genbank (NCBI), demonstrates a 100% sequence identity to *T. dicentrarchi*. Source water collecting during the AGD episode also shared some key taxa with the biopsy

samples, notably *Pseudoaltermonas*, *Propionibacterium* and *Tenacibaculum*.

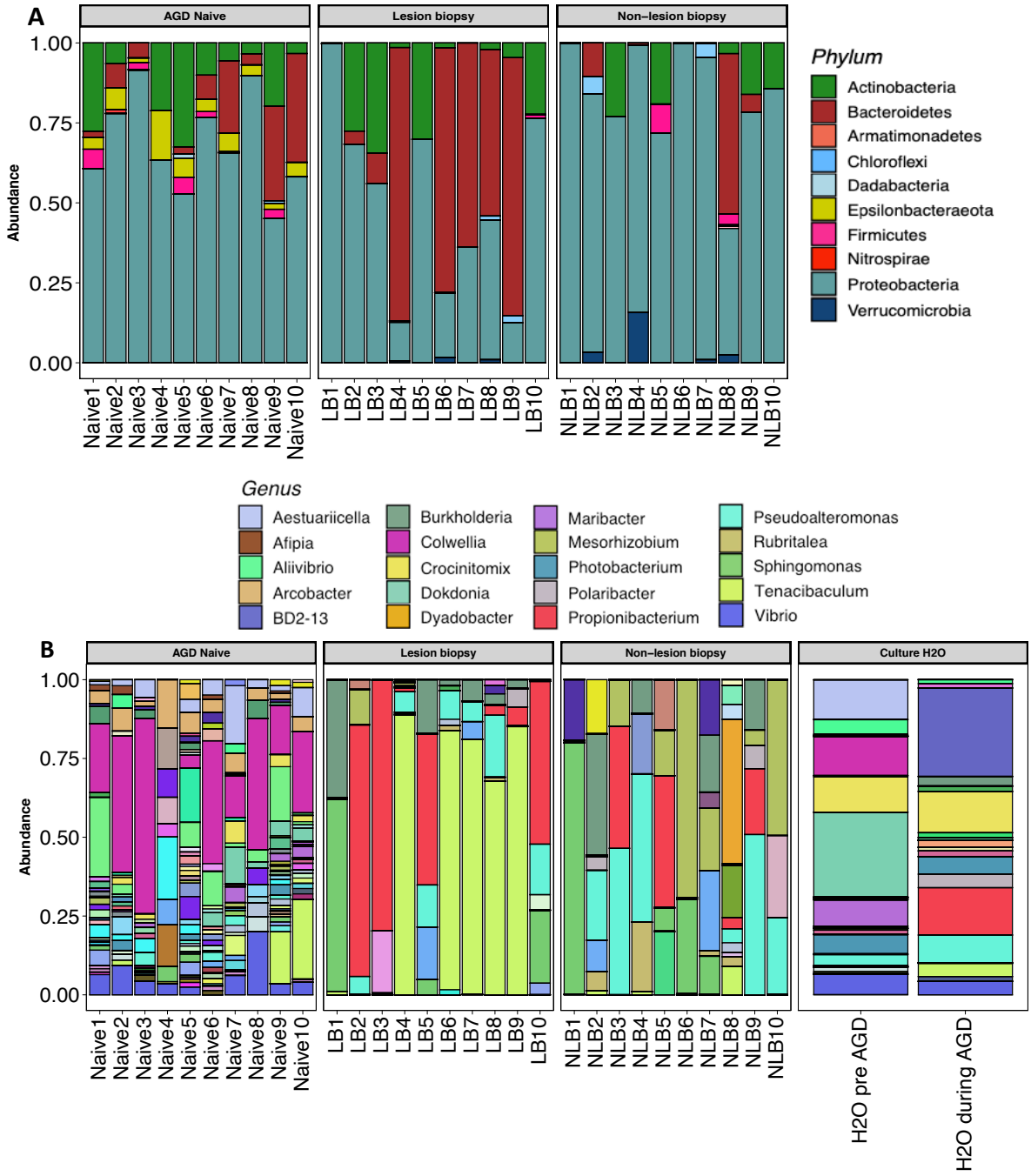

Figure 5.5. Microbial abundance of samples in this study. Plots show relative abundance (%) for Naïve, LB and NLB and fish at the phylum level (A) and Sterivex water samples at the genus level (B) level. Overall diversity of bacterial communities in the naive fish were higher, where AGD affected fish identified lower numbers of taxa with higher abundances.

The abundance of ASVs were agglomerated to the genus level and compared between groups using pairwise comparisons within a negative binomial general linear model. A total of 11 ASVs were deem significantly differentially expressed across our dataset. A significantly higher

abundance of ASV ID_748706, which was classified as *T. dicentrarchi*, was observed in the LB compared to the NLB and naïve groups (**Figure 5.6**).

From the taxonomic assignments to the genus level, we compared taxa which were differentially abundant between sample groups. Based on **Figure 5.6**, the analysis identified 11 assigned taxa (in addition to 'Other' grouped as the <1% abundant taxa) which were positively or negatively expressed between groups. *Tenacibaculum* and *Propionibacterium* were the dominant genus with the AGD affected gill tissue (LB group). Several taxa including *Arcobacter*, *Vibrio* and *Aestuariicella* were identified as positively expressed in the naïve group, but were in negligible numbers in both AGD positive sample groups.

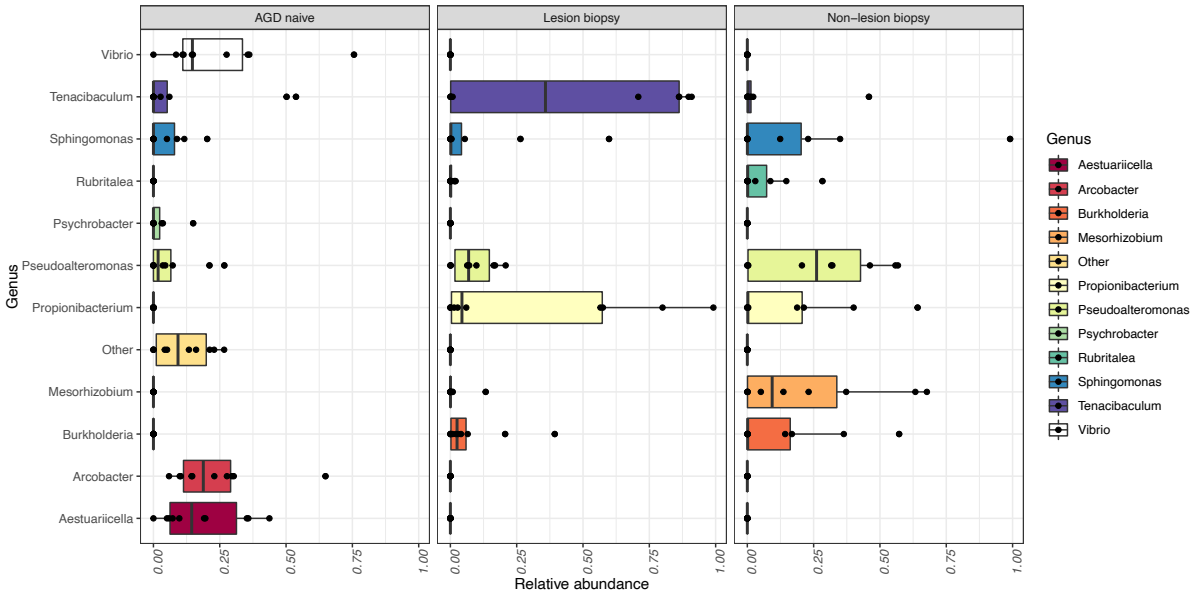

Figure 5.6. Top 11 genus differentially expressed between naïve, LB and NLB groups (DESeq2). The differential analysis was compared between all groups, with an adjusted p value of ($q<0.001$). Data shows that the taxa *Propionibacterium* and *Tenacibaculum* have a significantly stronger association to AGD lesioned tissue than in other groups.

5.4.3. Quantitative PCR

The abundance of *T. dicentrarchi* and *N. perurans* was further examined in the lesion and non-lesion biopsies using quantitative PCR. A relative standard curve analysis was performed and abundance presented as 18S or 16S copies for *N. perurans* and *T. dicentrarchi*, respectively. Lesion biopsies had a significantly ($p = 0.0115$) higher abundance of *N. perurans* 18S copies compared to non-lesion biopsies (**Figure 5.7A**). In agreement with the microbiome analysis we also observed an increased abundance of *T. dicentrarchi* 16S copies in the lesion biopsies. However due to the considerable fish-to-fish variation, the difference in *T. dicentrarchi* 16S copies between lesion and non-lesion biopsies was not statistically significant. A positive, but non-statistically significant

correlation between *N. perurans* 18S and *T. dicentrarchi* 16S copies was observed (Pearson; R = 0.38, p = 0.096).

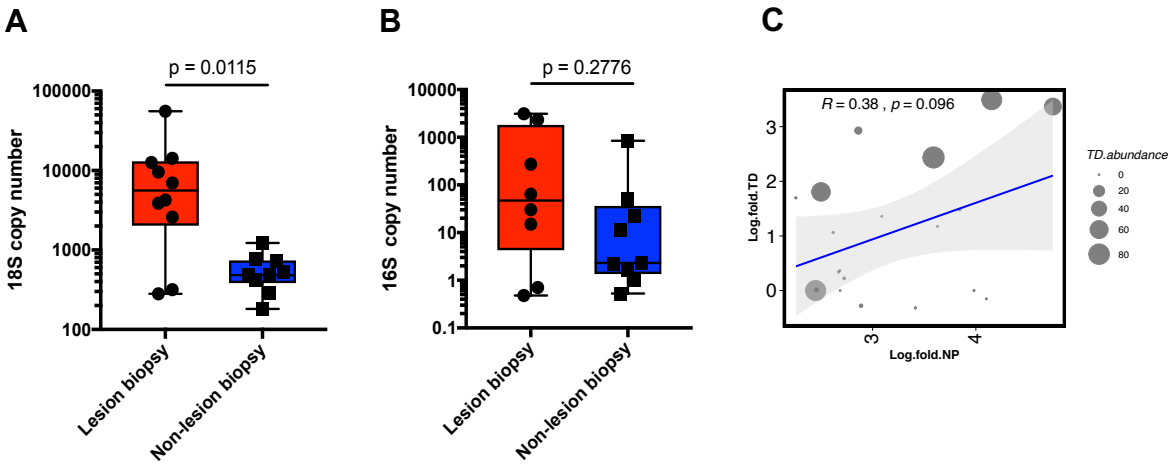

Figure 5.7. A - 18S gene copy number, and B- 16S gene copy number (B) for both *N. perurans* and *T. dicentrarchi* qPCR assays respectively, comparing LB and NLB sample groups (p value = Mann-Whitney non parametric test). C – Pearson correlation analysis of log transformed copy numbers of LB samples (overlaid with *Tenacibaculum* % abundance), suggesting a moderate positive relationship between N.P and T.dic loads on AGD lesion sites.

5.5. Discussion

AGD is a costly and detrimental ectoparasitic infection of salmonid species. Whilst the causative agent of the condition has been explicitly confirmed, only a few studies have examined the bacterial community in fish affected with AGD. The potential for host microbial dysbiosis that could influence AGD progression or increase disease vulnerability is to date largely unexplored. Understanding the bacterial community composition before and during an AGD episode as well as discrete recruitment to AGD affected gill microhabitats may facilitate further understanding *of N. perurans* pathogenesis.

Results from this study demonstrated that the bacterial community of the gill can be significantly altered following challenge with AGD. The observed bacterial taxa richness and diversity of AGD challenged fish was lower in biopsy samples containing branchial lesions. In contrast, salmon unexposed to *N. perurans* showed significantly higher bacterial richness and a more diverse and even community composition. Dysbiosis of teleost fish mucosal surfaces have been previously identified for a range of scenarios, whereby the commensal microbiome has been perturbed or compromised during infection. An *in vivo* challenge trial (Llewellyn et al., 2017), demonstrated that the skin microbiota of Atlantic salmon infested with the sea louse (*Lepeophtheirus salmonis*) was susceptible to colonisation from known pathogenic genera, including *Vibrio*, *Flavobacterium*, *Tenacibaculum* and *Pseudomonas*. It was also observed that over time, swab

samples from the whole skin surface were seen to decrease bacterial species' richness. Similarly, dysbiosis of the microbial community was also evident in Atlantic salmon skin following salmon alphavirus (SAV) infection (Reid et al., 2017) where bacterial community derived from a discrete skin sample indicated a global decrease in bacterial richness and diversity from low and high dose SAV groups at 14 dpi. Changes in the bacterial profiles were characterised by a decreasing abundance of *Proteobacteria*, and increasing colonisation of opportunist pathogens including *Flavobacteriaceae, Streptococcaceae* and *Tenacibaculum*. While it appears clear that both parasitic and viral infections can significantly reduce the microbial diversity in affected tissues, the consequence of dysbiosis upon disease pathology and the host response remains unclear. Microbial imbalance is commonly associated with pathogen insult, but disease treatment options can also significantly alter the microbial community, and potentially contribute to disease development and severity. Channel catfish (*Ictalurus puntactus*) treated with potassium permanganate led to a bacterial dysbioses of the skin and gills *in vivo* (Mohammed and Arias, 2015). Subsequent bacterial challenge of *Flavobacterium columnare* then resulted in significantly lower community diversity, and increased mortality for treated/challenged fish in comparison to non-treated/challenged counterparts. Other examples of gill co-infections and subsequent increased disease susceptibility are seen in complex gill disease (CGD). This is a gill condition associated with a number of different known agents, including *N. perurans*, Candidatus *Piscichlamydia salmonis, Desmozoon lepeophtherii*, salmon gill poxvirus and Candidatus *Branchiomonas cysticola* (Gjessing et al., 2019). It has been suggested that the primary pathogen/s compromise host immunity that leaves the gills vulnerable to colonisation by opportunistic pathogens (Herrero et al., 2018; Rozas-Serri, 2019). In the case of AGD, downregulation of critical immune pathways, including the major histocompatibility complex (MHC), has been observed within AGD branchial lesions (Young et al., 2008). This research suggests that immune function within the AGD affected tissue may be compromised which may ultimately contribute to the successful colonisation of pathogen bacterial taxa. Overall, data from our study corroborates with other published works demonstrating that the profiles of bacterial communities in gills are susceptible to alteration following pathological challenge.

Differential abundance testing showed significantly different genera between sampling groups. NLB samples contained a significantly higher abundance of *Pseudoalteromonas* and *Mesorhizobium*, whilst interestingly, branchial lesion biopsies had a much higher proportion of *Tenacibaculum* and to a lesser extent *Propionibacterium*. It has been shown that Atlantic salmon affected by AGD (with branchial lesions) are functionally compromised, with significantly lower aerobic scope, as well as decreased capacity to regulate homeostasis (Hvas et al., 2017). These impacts, amongst

others have downstream effects on appetite and immune vitality (Munday et al., 2001), and therefore waste excretion. Taxa which perform specialist biological functions readily colonise the healthy gill surface (Legrand et al., 2019; Lokesh and Kiron, 2016). For example, nitrogen fixing bacteria were linked to the expression of nitrogenous wastes at the gill surface in common carp (*Cyprinus carpio*) and zebrafish (*Danio rerio*) (van Kessel et al., 2016). In the instance of AGD hyperplasia where cellular tissue has lost excretory function, it is possible that this would ultimately impact the bacterial taxa from this functional group of taxa colonising such areas, affecting overall diversity. In the current study, NLB samples contained significant proportions of known nitrifying genera (*Mesorhizobium* and *Burkholderia*), which were not as abundant in LB samples specifically. In turn, *Propionibacterium*, a gram positive anaerobic taxa group, is commonly associated with skin and gland habitats, where they are able to effectively metabolise carbohydrates and carbon dioxide (Abdelsalam, 2017; Roy, 2011). *Tenacibaculum* have been identified as detrimental opportunist pathogens (Avendaño-Herrera et al., 2006), and proliferation of these taxa may be increased in the presence of functionally impaired gill tissue. *Tenacibaculum* (primarily belonging to *T. maritimum, T. dicentrarch*i or *T. finnmarkense*) are a group of emerging pathogens in global aquaculture, with several notable disease-causing species (Avendaño-Herrera et al., 2016; Frisch et al., 2018; Småge et al., 2018; Suzuki et al., 2001). A naturally occurring marine bacteria, it is usually horizontally transmitted in the water column and rapidly develops into an ulcerative disease of the skin, mouth, fins and gills, where extensive mats of gram-negative bacteria can cause significant tissue erosion and mortality (Avendaño-Herrera et al., 2006; Fringuelli et al., 2012; Wilson et al., 2019). The most significant change in composition in hyperplasic AGD affected gill material was the presence of an ASV classified to *Tenacibaculum dicentrarchi*, which was highly abundant (52.3-85.8%) in 50% of the sampled fish, and strikingly was differentially abundant between LB and NLB groups. This ASV was present at low to negligible levels in some of the naïve and NLB samples (along with *T. mesophilum* in the naïve group), although not in the high abundances seen in the LB group. Similarly, there was a negligible quantity of *T. dicentrarchi* present in the source tank water sampled both before and during the AGD challenge. Amplicon sequence data was supplemented by application of a *T. dicentrarchi* specific qPCR assay, confirming presence of the species in AGD affected fish. This assay also confirmed the presence and high quantity of 16S gene copy numbers relating to *T. dicentrachi* biopsies containing lesions suggesting that *T. dicentrarchi* were in greater abundance with diseased gill tissues in of AGD affected salmon.

Protozoan-bacterial coinfections have also been previously documented. At several Canadian farm sites, Arctic char (*Salvelinus alpinus*) and rainbow trout (*Oncorhynchus mykiss*) infected with

visible bacterial gill disease (*Flavobacterium branchiophilum*) were concomitantly affected with a nodular gill disease outbreak (Speare, 1999). While the exact nature of the co-infection was unclear, it was postulated that the amoebae present within hyperplastic lesions may have been a secondary infection. In a previous laboratory challenge of Atlantic salmon with *Tenacibaculum maritimum* which were sub-clinically affected by AGD, a noticeable increase was observed in mortality of co-infected fish compared to those only affected by AGD (Powell et al., 2005b). A longitudinal on-farm survey by Downes et al. (Downes et al., 2018) assessed several key salmonid pathogens, finding simultaneous qPCR pathogen load increases of *N. perurans* and *T. maritimum* on the gills of salmon during the grow out period. Confirmation of successful AGD induction in this study was completed using several gold standard diagnostics. Affected fish used in this study were moderately to severely affected by AGD based on individual gross gill scores (Taylor et al., 2009). Data from qPCR in this study indicates that both *N. perurans* 18S and *T. dicentrarchi* 16S copy numbers simultaneously increased at lesion sites and showed a moderate positive relationship between loads of each organism. The presence of *T. dicentrarchi* on branchial gill lesions found in this study may have manifested by a number of different mechanisms. It may have progressed from an opportunistic secondary infection source. Similar examples previously ascribed in the literature include (Llewellyn et al., 2017), where the skin microbiome of sea louse infected Atlantic salmon was altered to a more perturbed state, containing higher incidences of known pathogenic taxa, including *Tenacibaculum*. However, it is possible that the colonisation of *T. dicentrarchi* to compromised external tissues may be analogous to *T. maritimum* being conveyed in a trojan manner by an intermediate vector. This parallel has previously been drawn between *T. maritimum* and jellyfish species previously, where it was noted that during a farm outbreak that extensive *Phialella quadrata* blooms caused damage to Atlantic salmon gills, which was closely followed by an outbreak of tenacibaculosis (Ferguson et al., 2010). Bacterial sequence data from both jellyfish manubrium (mouth) and salmon gill samples showed a near identical resemblance of *T. maritimum,* indicating that the bacteria may be passing from the jellyfish to the gill, not as a separate opportunistic infection. Tenacibaculosis has also been characterised in Atlantic salmon net pen cleaner fish, lumpsucker (*Cyclopterus lumpus*) (Småge et al., 2016), also demonstrating a horizontal transmission source for pathogenesis. Precedence exists for amoebae to play the role of a transmission vector (Tosetti et al., 2014) , but has not been documented in *N. perurans.* Extracellular products (ECPs) have been identified previously as potential virulence factors for both *N. perurans* and the *Tenacibaculum* genus (Avendaño-Herrera et al., 2006; Bridle et al., 2015). Disintegration of the cellular epithelium is an important clinical sign of infection by *T. maritimum*, whereby potent exotoxins produced by the bacteria are able to stymie the host response. Toxicity

of *T. maritimum* extracellular products (ECP) has been demonstrated in Atlantic salmon previously (Van Gelderen et al., 2009). Potential exists for *N. perurans* to utilise the cytolytic effects of ECPs in initial attachment and pathogenesis, although this area is vastly unknown. In conclusion, this study found a significant decrease in bacterial diversity in AGD affected gill tissues and markedly so where biopsy samples contained branchial gill lesions. Additionally, this study observed a potential affiliation of *T. dicentrarchi* with AGD affected branchial gill lesions. *Tenacibaculum* sp., are a known secondary pathogen following host compromise via environmental, physical or pathogenic insult. *Tenacibaculum* sp. and potentially other virulent bacteria associated with *N. perurans* could play a role in the development of gill branchialitis in Atlantic salmon.

5.6. Acknowledgements

We gratefully thank CSIRO and Tassal Group Ltd for supporting this study. This research was enabled by a PhD scholarship awarded to JS from the Institute for Marine and Antarctic Studies (University of Tasmania). The authors wish to acknowledge Dr Richard Taylor for review of this manuscript and provision of fish for sampling, and to the salmon health facility technical staff (Chris Stratford and Lambertus Koster) for their contribution to husbandry of salmon stocks used in this study.

Chapter 6. General Discussion

6.1. Preamble

Amoebic gill disease (AGD) continues to be a prevailing health condition on salmonid mariculture operations globally. The aetiological agent of AGD, *Neoparamoeba perurans*, attaches to the gill surface of Atlantic salmon and elicits branchialitis that can be fatal. This pathogen-host interaction has been well-defined, however biotic factors affecting pathogenesis of AGD have not been fully explored. Previous studies demonstrate that the branchial surface is often co-colonised by a consortium of microbial agents in conjunction with *N. perurans*, which itself also harbours intracellular prokaryotic endosymbiont species. As such the possibility of a polymicrobial association which influences the pathogenesis of AGD remains possible, and largely unexplored. Bacterial species are localised in a community population on the gill surface in high abundance (Merrifield and Rodiles, 2015; Trust, 1975). However, a scarcity of comprehensive information exists regarding the relationship and interplay between parasitic amoebae and the branchial surface bacteria, which has guided and directed the research described within this thesis. The overarching research question of this research was to assess whether particular bacterial assemblages exert an influence on the pathogenicity of *N. perurans*. The major findings from previous chapters of this thesis have been synthesised and placed into current context to further knowledge of branchial microbial ecology of Atlantic salmon in relation to AGD, and more broadly in aquatic animal husbandry. These findings may assist future advancements in the field of AGD and gill diseases of finfish.

6.2. Distribution and diversity of gill bacteria

The scope for future bacteriomic gill studies is promising, with much information still to be gained from this area of research. It is beneficial for the wider research community to standardise appropriate sampling and analysis techniques, thus ensuring that the results and interpretations remain as comparable as possible. Findings from chapter 2 build our knowledge on an efficient, non-destructive and repeatable sampling technique while also increasing our understanding of gill microbiota dynamics.

Most notably, bacterial profiling across different hemibranch surfaces within individual fish showed a significant reduction in diversity of bacterial species upon the most posterior holobranch. Laboratory and field studies focussed on fish gill research commonly sample only one arch or common area on behalf of the entire gill basket in various diagnostics and assays. This is based on the assumption that one hemibranch surface is homogenously represented throughout the gill basket in terms of function and biological components. This research

described the potential for discrete regions or microhabitats on the gill surface, arising from bacterial community data obtained in chapter 2. While this concept has not been described in detail previously, supporting evidence including variable flow across hemibranch arches (Strother, 2013) or facultative blood perfusion activity in salmonids (Booth, 1978; Morgan and Tovell, 1973; Wood, 1974) may offer some explanation. Blood perfusion is the passage of blood flow distribution within the gill vasculature, which is a counter-current exchange between water and deoxygenated blood to meet metabolic demands (Roberts, 2012). It is postulated that fish can facultatively control factors such as blood flow to the most distal arch regions (i.e. lamellar extremities) (Ferguson, 2006; Roberts, 2012). An example scenario includes when fish are under low metabolic loads (such as resting), and only proximal portions of gill filaments may be perfused with blood (Nilsson, 2007). This capability assists with the efficiency of respiration, and helps avoid ventilation-perfusion mismatches. In the context of microbial agents colonising the gill, it is likely that areas of fully functioning lamellae (high cell circulation, excreting nutrients and ionic resources) may facilitate or promote a specialised range of organisms to colonise the mucus layer (e.g. nitrifying taxa) (van Kessel et al., 2016). Similarly, if posterior arches are subject to inconsistent or lower rates of perfusion, there may be a greater opportunity for pathogenic organisms to inhabit areas with less active defence mechanisms (i.e. circulating leukocytes). Inflammatory responses are achieved by bringing leucocytes to the site of tissue irritation, therefore tissue blood perfusion rates can likely determine the severity of inflammation (Ferguson, 2006). Another possible explanation for lower bacterial diversity in distal arch regions is the flow dynamics of water through the opercular cavity and the gills within. Water flows through the entire gill basket, but the dynamics between arches of differing size are not equal (Olson, 2002; Strother, 2013). The inference that flow fields may play a role in the lack of colonisation opportunities on the posterior arch surface is supported in the literature using methods such as quantification of parasites freely attaching to the surface of each arch. Indeed, several studies have postulated that water flow patterns within the gill may provide heterogenous parasite distribution patterns (Adams and Nowak, 2001; Forwood et al., 2013; Paling, 1968; Tripathi et al., 2010). It is possible that the posterior arches are simply exposed to less ventilatory flow volume, and subsequently have lower incidence of exposure to bacteria within the external waters.

Sampling methodology is therefore a key consideration for microbiome studies. Research in chapter 2 developed and validated methods to effectively characterise bacterial communities from surfaces of salmon gills, whilst comparing the effectiveness of different sampling and tissue preservation methods. Microbiota collected via non-destructive swabbing of the gill mucus and

wound generating biopsy of gill filaments was compared. Analysis revealed that the gill mucus swab was an adequate representation of the gill filament in terms of bacterial richness and diversity. This research has furthered knowledge regarding the impact of sampling methodologies in aquatic organisms. Prior studies available in this field offer consensus with this outcome, but have exclusively focussed on terrestrial or human subjects, which do not share the same fluid dynamics as the gill-water contiguity of aquatic species. For example, a swab of the sinonasal cavity of humans was deemed to be sufficiently representative of a tissue biopsy (Bassiouni et al., 2015). Another comparison of human skin sampling methodology demonstrated that collection via both eSwab and scrapes shared 99.3% of the 16S rRNA gene sequence reads (Bjerre et al., 2019). Similarly, a tape stripping method (to better standardise collection effort) was also comparable to a skin swab in the context of bacterial DNA collection (Ogai et al., 2018). In livestock pigs a similar comparison of swabbing versus tissue biopsy was undertaken by Hanshew et al. (2017), which found swabs to be a sufficient alternative to tissue-damaging biopsy when assessing microbial communities present. The results of chapter 2 were not only utilised in this thesis to facilitate representative gill microbiota collection for the research described in subsequent chapters, but can be applied more broadly for future gill microbiome studies. Substantial animal welfare benefits are also aligned to this result, where adequately robust sampling of the gill mucosa can be reliably obtained using non-destructive (non-fatal) methods. The nature of non-destructive sampling offers greater flexibility with experimental design, for example, temporally repeated measures studies of the gill bacteriome from the same individual. Any impact that gill swabbing may impart on the branchial bacteria community remains unknown. As such, future studies investigating this concept is warranted and would be valuable for experimental design.

Use of sample preservative or fixative is a crucial aspect of biological sampling regimes, as is it often impractical or impossible to process samples directly following collection. This means that generally the choice of fixative is dictated based on the subsequent analyses, although this may undermine the accuracy or completeness of microbial community samples when sequenced. Secondary to the swab-tissue biopsy comparison in chapter 2, seawater Davidson's (SD) fixative was compared against a commonly employed tissue preservative (RNAlater) to determine the impact upon bacterial DNA yield and sequence data. The rationale for this was to investigate the feasibility of using archived SD fixed gill filaments for retrospective bacterial profiling. Whilst formaldehyde-based fixatives such as SD solution are commonly employed for gill fixation, they are notoriously poor for the preservation of an intact mucus layer. This is likely due to hydration and removal of the mucus layer due to SD exposure (Leist et al., 1986; Lumsden et al., 1994),

which may have been partially or totally removed from the time that the sample was transferred to ethanol. Other less commonly applied fixatives such as methacarn were stated to retain the gill mucus layer more readily in histological fixation (Fernandez et al., 2019b), and may be an improved option for bacterial DNA preservation purposes. Further to this, results from chapter 2 concluded that that samples assigned for microbial profiling should be preserved using an accepted preservation method. Snap-freezing samples for later processing is widely accepted as a gold-standard sampling approach for microbiome studies (Pollock and Practice, 2018; Vogtmann et al., 2017). In this project, along with many others, access to such infrastructure and sampling regimes constraining instant laboratory processing meant that snap-freezing samples was not feasible. However preservatives such as RNAlater are also deemed to be sufficient for sample storage (Flores et al., 2015; Franzosa et al., 2014; Nechvatal et al., 2008; Sinha et al., 2016; Song et al., 2016; Voigt et al., 2015). Numerous studies have compared bacterial diversity upon tissue samples fixed in preservative solutions with the same tissues that were preserved fresh or snap frozen. Results across such studies are variable but generally it appears that some level of diversity is lost after using sample preservative products such as RNAlater, OMNIgene, or ethanol (Choo et al., 2015; Pollock and Practice, 2018; Vogtmann et al., 2017). The general consensus is that the use of 70% ethanol as a sample preservative has the greatest impact upon bacterial diversity, and as such is not recommended for this work (Song et al., 2016; Wang et al., 2018).

Anaesthetic use is another husbandry procedure that could potentially affect microbiome studies. From a welfare and animal ethics perspective, anaesthesia of fish is essential to safely handle, euthanise and sample individuals (National Health and Medical Research Council, 2013). All fish related procedures carried out in data chapters 2, 3, 4 and 5 were completed using AQUI-S, a compound synthetically derived to mimic the active from clove oil (iso-eugenol) as an active ingredient. This solution has been documented to have some microbicidal properties in the literature, where usage at high concentrations can cause significant trophozoite detachment of *N. perurans* (Shijie et al., 2016), and bactericidal effects on colony counts from 20-60 ppm (Al-yaqout et al., 2014). It is noted that the normal working concentrations and exposure times for *in vivo* and *in vitro* work are unlikely to have significant antimicrobial (bacteria or amoebae) properties, and in the event of bacterial cell lysis, the resultant DNA is likely to be captured and profiled. Given the animal welfare considerations for fish anaesthesia usage of AQUI-S was deemed an unavoidable limitation for this work, but was applied at a standardised exposure rate across all studies completed.

We also demonstrated that branchial bacteriomic profiling could be restricted to discrete biopsies of hyperplastic gill lesions in chapter 5. Sampling exclusively within these lesion microhabitats gave a clear distinction of *Tenacibaculum* abundance between AGD affected and non-affected lamellae. In similar AGD affected cohorts in chapter 4, a surface swab of 8 hemibranch arches was also able to identify *Tenacibaculum* among the bacterial consortia. However, this sampling regime was not capable of determining the lesion-specific abundances with clarity to infer the results observed from discrete targeted biopsies in chapter 5. This serves as an example that optimised sampling protocols need to be developed for each unique research question, and can be the differentiating factor between gaining insights and useful results in bacteriomic studies.

6.3. Did antibacterial modulation affect the gill microbiota?

The central aim of this thesis was to establish whether alteration to gill associated bacteria would impart an effect upon the onset and progression of AGD. Chapter 3 describes the formative aspect of this research, which involved selecting suitable antimicrobial agents and screening those candidates *in vivo*. A range of oxidative immersion bath and orally administered antibiotics were used in this study to investigate their antimicrobial properties. Such antimicrobial compounds are commonly used in aquaculture to treat a range of topical conditions and pathogen outbreaks in fish. The broad-spectrum nature of these compounds may negatively affect commensal bacterial although published evidence is lacking. Comparison of the branchial bactericidal effect of several agents was critical to obtain reliable methodologies to be employed for later studies.

Culturable bacterial loads were reduced significantly on the gill surface based on data from chapter 3 and 4, where both oxidative chemical bathing and orally administered antibiotics imparted a bactericidal effect at the branchial surface. The best performed immersion bath and oral antibiotic in terms of bactericidal effect were chloramine-t and oxytetracycline (OTC) respectively. Similar results have been observed in a culture dependent study where Atlantic salmon were exposed to potassium permanganate (Embar-Gopinath et al., 2005b). While culturable loads on the gill decreased after antimicrobial intervention in the current studies, it was notable that the subsequent taxa richness from respective amplicon sequence data of the same samples did not decrease in-kind. This suggests that while culturable species may be reduced after treatment, it is likely that other treatment-resistant taxa colonise in their place. Such information has not been previously described in this scenario, and these results are pertinent to consider for future NGS studies or indeed any other culture dependent investigations. Interestingly the in-feed antibiotics (florfenicol and oxytetracycline) provided longer lasting bacterial reductions in terms of culturable taxa. They also showed less pronounced taxonomic

alterations in post-treatment bacterial community profiles comparative to oxidative bath compounds (relative to the respective control treatments) used in these two studies. Such examples of antimicrobial treatments eliciting downstream impacts on commensal bacteria are becoming more understood. For example Minich et al. (2020) completed an assessment of gill bacteria in Southern bluefin tuna (*Thunnus maccoyii*) treated with the antiparasitic praziquantel, revealing a significant reduction in gill bacterial diversity and richness. Results from chapter 3 showed that exposure of the gill to hydrogen peroxide (in both fresh and saltwater baths) had the highest impact upon taxonomic community structure. Histologically, it has been observed that hydrogen peroxide can cause superficial damage to gill lamellae surfaces when concentration or exposure times are excessive (Kiemer and Black, 1997; Wynne et al., 2020a). While gill damage was not assessed in this study, the peroxide concentrations tested were within safe working parameters reported from other published works (Adams et al., 2012; Powell et al., 2005a; Wynne et al., 2020a), and no abnormal gross signs were detected in the gills. The perceived higher risk of lamellar damage from hydrogen peroxide outlined in literature, coupled with the slightly inferior post-treatment bactericidal effect meant that chloramine-t was selected as the immersion bath treatment for future trial usage in chapter 4. In summary, this work has advanced our knowledge around superficial and systemic antimicrobial treatments. The action of being able to reduce culturable bacteria has again been demonstrated as per previous studies, although further insights into community structure are now available. These results indicate that the microbiome reacts dynamically to alterations, and that other treatment resilient or opportunistic pathogens can colonise these spaces.

Observations made throughout this project demonstrated a vast fluctuation of gill microbiota after fish handling or movement between systems. This suggests a better understanding of the impact of regular aquaculture husbandry activities on fish health is warranted. During the initial setup of experiments in chapters 3 and 4, it was observed that significant disturbances to the bacterial profile may have originated from fish handling and habituation into new tank systems. Manual handling of Atlantic salmon in a study by Minniti et al. (2017) revealed that one discrete hand-netting event led to a rapid and distinct change in community structure over a 24 hour period. Results from chapter 3 indicate that the timeframe to microbial stabilisation is likely much longer, with un-treated, unchallenged control fish requiring upwards of 3 days post handling to maintain a relatively stable gill microbiota population. This result is somewhat confounded under the auspices of transferring between tank systems (RAS holding cohort to flow-through experimental replicate tanks), where batches of fish were held in larger numbers for smoltification prior to being stocked into systems. Nonetheless, understanding the microbial

impact arising from mechanical, environmental or disease-based stressors will lead to improved production practices. Other examples of perturbations including environmental stress or disease can lead to commensal bacterial community dysbiosis (Egan and Gardiner, 2016). For example, acute cold-water stress applied to the late egg developmental stage significantly affected the gut and skin community of larval Atlantic salmon (Webster et al., 2020). Similarly, seawater transfer of Atlantic salmon smolt caused an appreciable transition of the microbiota occupying the skin mucus (Lokesh and Kiron, 2016). Production-based stressors on biological culture conditions can also lead to a disturbance of commensal bacteria, potentially increasing the chance of pathogen outbreak. Suboptimal culture conditions arising from high density production may also affect how bacterial colonise mucosal surfaces. For example, a recent study by Mota et al. (2019) observed that Atlantic salmon exposed to a CO_2 concentration greater than 19 ppm in recirculating aquaculture (RAS) systems developed a thinner skin epidermis layer, which could potentially render the host more susceptible to pathogen infiltration. A notable finding from this component of research was that the gill bacteria community dynamics undergo rapid change and population structure in the presence of stressors. The combination of handling, system movements and treatments may be impacting health of fish immensely and understanding the microbial impact and recovery times may help to alleviate such issues in aquaculture enterprises. Chapters 3 and 4 demonstrated that water-borne microbiota can play a role in gill surface colonisation, and that crosstalk between gill mucus and water during sampling is likely to have occurred. In chapter 4, sampling of water from the fish-holding RAS revealed a significantly different bacterial profile to the experimental tank array with flow-through seawater. Taxonomic analysis of water samples from the holding RAS indicated that low abundances of *Rubritalea* (phylum *Verrucomicrobia)* corresponded with low colonisation levels in the gill mucus samples collected initially (Time 0). This taxon became highly prevalent in a majority of gill mucus samples at 11 dpi and 21 dpi which can be linked to the source flow-through seawater community in the experimental array. Bathing with oxidative chemicals such as hydrogen peroxide demonstrated that a large proportion of the gill-based bacterial taxa (distinct from water-based taxa) were removed from the mucosal surface. Source population analysis (FEAST) was able to demarcate core microbiota originating from tankwater and from gill mucus respectively. Comparing the relative abundance of each core sample type showed that significantly higher proportions of water-based taxa colonised the gill immediately post hydrogen peroxide exposure. Mucosal integrity may have been impacted by chemical oxidation and mucus layer hydration (in the freshwater treatment). It is possible that fish responded to the chemical insult by rapid production of mucus (Powell and Perry, 1996) (and therefore high rates of

shedding), which sloughed the majority of bacterial taxa from this area. Loss of gill commensal bacteria may have detrimental effects to the host in terms of immunocompetence and function, although determining the origin of bacteria as water-based or gill-based remains difficult using standard sampling methods. Future studies would benefit from further characterising the impacts (negative, or otherwise) from a loss of gill-associated taxa from the branchial surface.

6.4. The relationship between AGD progression and microbiota of the gill

Previous studies characterising how the gill microbiota affects AGD development are sparse and limited in scope. Chapter 4 examined the progression of laboratory induced AGD in Atlantic salmon, giving novel insights as to how the branchial bacterial community responds following AGD exposure, and also the effect of bacterial modulation (via antimicrobial treatments) upon the course of an AGD challenge. Critically, AGD was able to progress in fish that were orally or topically administered OTC and chloramine-t respectively through 11 and 21 dpi. This provides further substantiation of *N. perurans* as the primary aetiological agent of AGD (Adams and Nowak, 2004; Crosbie et al., 2012; Munday, 1986; Roubal and Lester, 1989; Young et al., 2007; Zilberg and Munday, 2000), and that disease onset can occur commensurate with lowered levels of gill bacteria (Embar-Gopinath et al., 2005a). We observed changes in the bacterial community 21 dpi after infection with *N. perurans* in chapter 4, however the alpha diversity metrics did not decrease as drastically as other examples of affected finfish during the course of disease (Legrand et al., 2018; Li et al., 2016; Llewellyn et al., 2017). The visual signs of AGD described in chapter 4 were less severe in the positive control group (mean gill score 2.72) than those observed in gill samples described in both chapter 2 and 5, (mean gill score 3.20 and 3.30 respectively). From this perspective, examining the specific gill microhabitats of 'lesioned' and 'non-lesioned' gill lamellae in chapter 5 it was observed that bacterial diversity was significantly lower in AGD affected individuals, as opposed to naïve (unchallenged) fish, but critically, diversity was lowest upon excised hyperplastic filaments compared to adjunct normal gill filaments. A study by Botwright et al. (2021) completed in parallel with chapter 5 of this thesis employed a dual-RNAseq approach to investigate the host-parasite interaction model in AGD. The upregulation of genes aligned to immune response mediation, cellular proliferation and invasion provisioned an upregulation of the overall local immune response on the gill. This supports previous evidence that impacts and host responses arising from AGD are predominately localised within focal lesions (Morrison et al., 2006b; Wynne et al., 2008; Young et al., 2008). It is thought that because AGD causes proliferative lamellar hyperplasia and superficial necrosis of the gill mucosa, such largescale changes to the gill infrastructure it is likely some level of functionality is impaired. This may lead to alteration of the bacterial community present, given the lack of excretory by-

products available as a resource (van Kessel et al., 2016), or the absence of commensal bacterial function to exclude opportunists (Ross et al., 2019). This data collectively demonstrates that severe AGD contributes to a decrease in microbial diversity.

Taxonomically there was an appreciable shift in the dominant genus which colonised the gill surface at 21 dpi in chapter 4. Several clades of bacterial taxa were identified that are regularly implicated in finfish health conditions either as a primary agent of disease, or by contributing to opportunistic or secondary infection following a disease event. Increased abundance of *Tenacibaculum*, *Aliivibrio*, *Vibrio*, *Aeromonas* and *Pseudomonas* was observed in AGD affected fish in our studies (chapters 2, 4 and 5). These taxa are all capable of causing disease in many aquatic scenarios (Austin and Austin, 2016; Noga, 2010), but are also found at low to negligible levels upon fish mucosal surfaces in normal conditions. The development or increased abundance of potentially pathogenic taxa are characterised as the pathobiome (Sweet and Bulling, 2017; Vayssier-Taussat et al., 2014). Recently, the increased proportion of the pathobiome within the microbial community as a direct result of disease or stressors has become more apparent (Bass et al., 2019). Results from our studies indicate that AGD promotes proliferation of the pathobiome on the gill mucosa. This finding can be placed within the context of other disease conditions of finfish which experienced similar post-infection impacts including sealice infestation (Llewellyn et al., 2017), *Flavobacterium* infection (Mohammed and Arias, 2015) and *Tenacibaculosis* (Miyake et al., 2018). This line of investigation is crucial to determine the impact that these genera have upon gill health.

Components of this work have increased knowledge on the microbial dynamics and impact on AGD progression. Atlantic salmon exposed to chloramine-t demonstrated a higher aggregate gill score, increased percentage of AGD affected filaments and a higher *N. perurans* burden (by qPCR). Concurrent to this, the bacterial community on the gill after chloramine-t treatment was dominated by potentially detrimental taxa including *Staphylococcus* (post treatment), *Tenacibaculum* (11 dpi) and *Aliivibrio* (21 dpi). This incremental increase in AGD severity may have been due to an immunosuppressive effect that treatments with oxidative chemicals have been reported to impart in fish. Treatment with chloramine-t removed much of the commensal microbiota, which may have impacted the innate commensal function upon the gills. Atlantic salmon exposed to chloramine-t in Genaro Sanchez et al. (1997) were shown to harbour *Tenacibaculum* and *Pseudomonas* in their skin mucus layer. Rainbow trout (*O. mykiss*) treated with chloramine-t also appeared to lose some non-specific immune function such as lysozyme serum activity (Yavuzcan

Yildiz et al., 2009). Chloramine-t is a known gill irritant, which may have the potential to exacerbate disease signs, although we did not observe any obvious gross gill irritation in the chapter 3 study where fish were exposed to chloramine-t but not challenged with *N. perurans*. It is not possible to differentiate whether the chemical immunosuppression, commensal defence removal, or a combination of both (or other) factors played a more prominent role in the more severe disease state observed.

Previous research investigating the role of culturable bacteria on AGD affected fish showed that the amount of lesion affected filaments increased when Atlantic salmon were concurrently challenged with *N. perurans* and *Winogradskyella* sp. (Embar-Gopinath et al., 2006). It remains unclear whether the increased bacterial abundance exacerbated the superficial gill necrosis and hyperplasia, or if the increased bacterial load created a favourable environment for amoebic proliferation in the water column or upon the gills. The *Winogradskyella* strain used was reported as non-pathogenic to fish, and unlikely to be a food source of *N. perurans* (Embar-Gopinath, 2006). The authors concluded that exposure to this bacterium may have increased the susceptibility of Atlantic salmon to *N. perurans*. It was also observed that AGD affected smolt had a higher proportion of bacteria originating from the Cytophaga-Flavobacterium-Bacteroides (CFB) group (Embar-Gopinath, 2006). A previous field study similarly demonstrated that smolt stocked into seapens had an increase in culturable CFB colonies from the gills which increased in magnitude as AGD progressed (Cameron, 1993). An opportunistic assessment of bacteria associated to *N. perurans* and the focal gill lesion was completed to determine some key microbial components as a byproduct of dual-RNAseq transcriptomics (Botwright et al., 2021). These results demonstrated that order *Flavobacteriales* was a dominant feature on the gill, with another potentially pathogenic species, *Nocardia jejuensis* implicated in the AGD lesion filaments. *Tenacibaculum* are a member of the *Flavobacteriaceae* group, and it is possible that the increase in CFB counts described above may be a linkage to *Tenacibaculum* abundance as AGD progressed, or indeed to *Psychroserpens* (also from the *Flavobacteriaceae* family), which was also associated with AGD affected individuals (Bowman and Nowak, 2004). Other field-based data from AGD affected farmed salmonids between 2015 and 2019 have shown high prevalence of *Tenacibaculum* sp. present via both microbial profiling and qPCR amplification (Wynne, unpublished data).

6.4.1. Evidence for microbial interaction between *Neoparamoeba perurans* and *Tenacibaculum dicentrarchi* in AGD development

By sampling discrete biopsies of AGD affected smolt in chapter 5, the bacterial community profiles of AGD affected gill filaments were compared against adjacent, unaffected filaments and

gill filaments from naïve (unchallenged) fish. Taxonomic results demonstrated that 50% of the cohort sampled for AGD lesion biopsies contained a *Flavobacterium*, *Tenacibaculum dicentrarchi* in very high abundance (52.3-85.8% of total community). Such a stark contrast between the lesioned tissue samples and the very low abundance of *T. dicentrarchi* on the adjunct-unaffected biopsies (mean <1%) provided some evidence toward a potential role for pathogenic bacteria during AGD development. These two pathogenic agents have been identified concurrently in a range of scenarios. A culture independent study by Bowman and Nowak (2004) identified two clone sequences of *Tenacibaculum maritimum* within a small proportion of laboratory challenged AGD affected gill filaments. Longitudinal screening of Atlantic salmon demonstrated that coinfections of *T. maritimum* and *N. perurans* were consistent within the farm environment (Downes et al., 2018). *Tenacibaculum* (formerly known as *Flexibacter)* are a clade of highly pathogenic taxa, emerging as one of the most concerning bacterial finfish diseases globally (Avendaño-Herrera et al., 2006; Grothusen et al., 2016). Due to the ambiguity of species level resolution in 16S amplicon sequencing it was important to provide supplemental diagnostics to confidently identify the species of *Tenacibaculum* present. To achieve this a species-specific quantitative PCR assay for *T. dicentrarchi* was designed in the absence of a suitable published assay. This assay confirmed the presence of the bacteria at high levels, in agreement with amplicon sequence data. Copy number abundance of the 18S *N. perurans* gene also increased in concert with the 16S *T. dicentrarchi* gene, giving a moderate positive correlation of the two organisms. Quantitation by relative curve analysis suggests that the two organisms were increasing in abundance concurrently, which may be linked to the development of more severe gill pathology or mature lesions.

Tenacibaculum dicentrarchi has been previously documented to induce tenacibaculosis in finfish species including seabass (*Dicentrarchus labrax*) and salmonids (Klakegg et al., 2019; Piñeiro-Vidal et al., 2012), where skin ulceration and fin rot ultimately lead to high mortality. In the chapter 5 study, AGD affected Atlantic salmon were shown to have no gross external pathology indicative of tenacibaculosis despite the high abundance of *T. dicentrarchi* associated with gill lesions. It possible that *T. dicentrarchi* colonised the gill lesion surface post amoebae attachment as an opportunist, though this remains unknown. Opportunistic colonisation of bacterial and fungal agents has been previously described following amoebiasis-mediated tissue compromise (*Entamoeba histolytica*) in human health (Ghrenassia et al., 2017). It is possible that compromised gill mucosa favours infiltration by *T. dicentrarchi*, and that such bacteria may be attracted to these areas by the loss of regular host defence (Cheng et al., 1981). AGD lesions are highly demarcated and typically feature extensive cellular proliferation and mucus production. Histologically these

lesions are characterised by epithelial hyperplasia, lamellae fusion and oedema (Adams and Nowak, 2001). At the immunological level pro-inflammatory cytokines, including Interleukin-1 beta, are also up-regulated within AGD lesions (Morrison et al., 2007). Therefore, a probable scenario is that *T. dicentrarchi* is an incidental environmental organism colonising immunocompromised gill regions.

It remains possible that *N. perurans* may harbour or transport *T. dicentrarchi* in a trojan horse style mechanism as demonstrated for other amoebae genera (Guimaraes et al., 2016). Data from chapter 4 suggests that the inoculum used to expose fish to *N. perurans* also contained several unique ASVs (representing potentially pathogenic taxa such as *Tenacibaculum*) which were present on the gill surface post-inoculation. In such a scenario, the potential variance between intracellular or trophozoite-affiliated bacteria may better explain the variable rates of *T. dicentrarchi* colonisation between individual gill lesion samples observed. The aggressive pathogenic nature of the *Tenacibaculum* genus raises the possibility that *N. perurans* could use *T. dicentrarchi* as a virulence factor in gill branchialitis. Extracellular products (ECPs) are cytolytic compounds secreted by pathogenic organisms to assist in degradation of organic materials and inhibit cellular functions (Austin and Austin, 2016). *Tenacibaculum* are known to produce and utilise the effect of ECPs as an infection strategy (Avendaño-Herrera et al., 2006; Van Gelderen et al., 2009). *N. perurans* also possess ECP production (Bridle et al., 2015; Cano et al., 2019), but it remains unclear whether *N. perurans* could utilise cytolytic ECPs from other organisms (or produce ECPs via phagocytosis of pathogenic taxa) as a means to proliferate on the gill epithelium more effectively. Necrotic factors associated with both *N. perurans* attachment and *Tenacibaculum* infiltration require further elucidation to determine the impact or effect. Amoebic infections of other higher order vertebrates such as *Acanthamoeba* keratitis are thoroughly investigated and can provide insights into host-amoebae-bacteria interactions. In pathogenesis of keratitis, *Acanthamoeba* trophozoites bind to the corneal surface via mannosylated proteins which often develop as a result of abrasions to the cornea (often via use of contact lenses) (Niederkorn, 2021). This process engages trophozoites to secrete mannose-induced protease MIP-133 which lyses both corneal cells and Bowman's membrane, furthering tissue damage and severity of the condition (Neelam and Niederkorn, 2017). The severity of this process can be increased by the presence of certain microbial factors. For example, the bacterium *Corynebacterium xerosis* was observed to significantly increase the rate of trophozoite attachment to the ocular surface. This bacterium contains a highly mannose-rich cell wall which facilitates greater production of MIP-133 (Niederkorn, 2021). This is a well-defined relationship, but in terms of amoebic-bacteria cofactor virulence, it is one of a limited number detailed with such assurance.

Similar bacterial-amoebic complexes exist in freshwater aquaculture, where nodular gill disease (NGD) poses health issues for salmonid species (Buchmann and Nielsen, 2004; Daoust and Ferguson, 1985). The causal amoebae were thought to be *Cochliopodiidae*, but evidence suggests that a multi-amoeba aetiology may be characteristic of this condition, and include *Amoebozoa*, *Naegleria* sp. and *Rhogostoma minus* (Dyková et al., 2010; Dyková and Tyml, 2016; Noble et al., 1997; Speare, 1999). Interestingly, the prevalence of NGD is often linked to a serious bacterial pathogen, *Flavobacterium branchiophilum*, the causal agent of bacterial gill disease (BGD). It was noted in Speare (1999) that a concomitant infestation of NGD occurred in farmed Arctic char and rainbow trout affected by *F. branchiophilum*. The amoebae appeared to become prevalent directly post-bacterial infection, a scenario also observed by Bullock et al. (1994). As both studies reported the outcome of unplanned disease outbreaks, no assessment or quantitation could be made regarding whether NGD was exacerbated by the presence of *Flavobacterium*. Both studies noted that amoebae trophozoites may have been attracted to the gill by the high abundance of bacteria, and that extensive hyperplasia was rapid and severe (Bullock et al., 1994; Speare, 1999). It is also possible that amoebae presented normally on the gill surface, and the bacterial invasion predicated the ability of the trophozoites to proliferate. The infection complex between BGD and NGD requires further elucidation, but supports the overall hypothesis that known pathogenic agents can alter the phenotype of ubiquitous organisms and promote a potentially pathogenic response.

Regardless of the exact machinations of *N. perurans* and *T. dicentrarchi* within gill lesions, this result demonstrates that the phenotype of the bacterial community can be altered as a result of AGD progression. Interestingly, *T. mesophilum* along with *T. maritimum* and *T. dicentrarchi* were found in the background community of apparently healthy AGD naïve stock in chapters 2, 3, 4 and 5, indicating that *Tenacibaculum* is likely part of the ubiquitous consortia of microbes in the marine environment. It was also noted that *T. dicentrarchi* abundance was low to negligible in water filter samples. This result was mirrored in Wynne et al. (2020b), where Atlantic salmon affected by yellowmouth (*T. maritimum*) also had negligible pathogen load in seapen water, but extremely high abundances in the jaw cavities of affected smolt. In this context *T. dicentrarchi* may act with autonomy from any AGD event in the laboratory system used in these trials, existing at negligible levels unless triggered into a pathogenic response either by the host or the environmental conditions changing into a state that favours infiltration or proliferation. It is important to note that all gill mucus samples from AGD affected fish in this project (chapters 2, 4 and 5) contained the *Tenacibaculum* genus. This result may also warrant consideration in the context of a multi-pathogen complex, similar to that seen in the northern hemisphere, where

complex gill disease (CGD) and proliferative gill inflammation (PGI) share multiple primary agents (Boerlage et al., 2020; Herrero et al., 2018; Rozas-Serri, 2019; Steinum et al., 2010). Within these conditions both *N. perurans* and *Tenacibaculum* spp. are frequently identified in co-abundance on the gills of Atlantic salmon (Downes et al., 2018; Gjessing et al., 2019). Other ectoparasitic infections of finfish have demonstrated significant co-factor interactions to bacterial agents at the host-pathogen interface. It was observed that the mechanical damage on the skin of goldfish (*Carassius auratus*) caused from *Ichthyophthirius multifiliis* accelerated infection of *Aeromonas hydrophila*, and that Ich may act as a reservoir for the bacteria (Liu and Lu, 2004). The same parasite-bacteria relationship examined by Xu et al. (2012) demonstrated that channel catfish (*Ictalurus punctatus*) mortality was significantly increased when exposed to both pathogens. *Flavobacterium psychrophilum*, a common bacterial disease of hatchery reared salmonids was found to be significantly enhanced by the presence of *Gyrodactylus derjavini* (Busch et al., 2003), where fish that were unexposed to the ectoparasitic monogenean largely remained unaffected *in vivo*. Bandilla et al. (2006) demonstrated that mortality in rainbow trout (*Oncorhynchus mykiss*) exposed to fish lice (*Argulus coregoni*) and *Flavobacterium columnare* were significantly higher than fish challenged with only the parasite or bacterial agent alone. These results emphasise the significance of concomitant pathogen exposure, and possible virulence enhancement of bacterial infections resulting from ectoparasitic invasion.

6.5. Avenues for future investigation

This project has defined and profiled the branchial bacterial assemblages of Atlantic salmon in greater detail than any previous works in this area. Identification of specific bacterial taxa associated to AGD progression was a crucial step to better understand the microbial ecology of the disease. This knowledge has shed further light on microbiota of the gill and in the presence of *N. perurans*. To gain a comprehensive understanding of the impact of non-amoebae microbiota in AGD, the outcomes of these studies need to be augmented with further investigations. Some of these potential pathways are detailed and discussed.

An obvious aim for future research is to elucidate the functional role of the identified AGD-affiliated bacteria (i.e. *T. dicentrarchi*) or other pathogenic bacteria across different infection environments, to quantify potential virulence enhancement of *N. perurans*. This information would likely underpin a pathway to understanding pathogenesis of AGD at a similar level ascribed to well-defined amoebic diseases of higher vertebrates (e.g. *Acanthamoebae* spp. and *Entamoeba histolytica*). Future research in this area should be directed toward gaining greater understanding of the cause-and-effect of the pathogenesis (i.e. whether there is underlying

linkages or a complex / concurrent infiltration process). Potential identification of microbial co-factors could lead to research into mitigation of this on the gill surface. Applicable solutions could include vaccine development or targeted phage therapy to remove pertinent bacterial species and re-assess the amoebic challenge system. The development of vaccines for *Tenacibaculum* species is currently under development in several countries, which may provide further understanding to any role that the bacteria may play in AGD.

The pathobiology of *N. perurans* is not fully characterised, which continues to cause ambiguity in pathogenesis of AGD. Findings from this research indicate that effort to investigate the trojan horse model theory is worthwhile. Identification of bacteria within the cytoplasm of amoebae species (Dyková and Lom, 2004) alongside recent investigations confirming the presence of intracellular *Vibrio* sp. within *N. perurans* trophozoites (MacPhail et al., 2021) provide justification for renewed research efforts. Isolation and true axenic *in vitro* culture of *N. perurans* in a sustainable fashion still eludes the AGD research community. This project utilised wild-type *N. perurans* exclusively however access to axenic *N. perurans* would be an incredibly informative tool to investigate microbial relationships and interplay. Such a capability may allow the microbial ecology to be fully modulated, and provide the option to culture *N. perurans* in the presence of potential virulence enhancing organisms. Subsequent exposure to cell monolayers or targeted *in vivo* studies could then proceed in much the same fashion as previously well-described amoebic infections such as *Acanthamoeba* spp.

Several important research topics have been raised around mucosal barrier health, in particular the proliferation of a pathobiont in chapters 3 and 4. While antibacterial treatment prior to *N. perurans* challenge did not inhibit AGD progression, potential future work could examine post-AGD challenge antibacterial exposure. Timing of the treatment may play a greater role in removing trophozoite-associated bacteria and potentially affecting virulence mechanisms. Results in chapter 4 suggested the development of a significant pathobiome phenotype arising from antibacterial treatment and *N. perurans* challenge. Future studies in this area may benefit from investigating whether low bacterial diversity or pathobiome phenotypes (caused by perturbation) actually predispose or increase the susceptibility of fish to AGD assessed over a longer timeframe, or if the proliferation of these taxa is only a downstream symptom of AGD onset. Furthermore, dedicated integration of advanced 'omics' techniques such as RNA sequencing (transcriptomics), protein expression (proteomics) and small molecules of the organism (metabolomics) could infer functional components of the bacterial community, a capability that

is limited in 16S amplicon sequencing.

Whilst out of scope for the aims of this thesis, methods for mitigating against microbial dysbiosis from antimicrobial treatment may be crucial, and this could include the use of probiotic additives. Limited examples of fish-based probiotics to mitigate disease exist in the literature, however use of probiotics after streptomycin exposure demonstrated that mortality was reduced significantly compared to an antibiotic only treatment (Schmidt et al., 2017). Similarly, (Schubiger et al., 2015) demonstrated that *Enterobacter* (C6-6) administered orally in-feed provided protection against cold-water disease in rainbow trout (*Oncorhynchus mykiss*) after intraperitoneal injection with *Flavobacteria psychrophilum*. Development of a novel gill-based probiotic (or administration of a commercial product) could prove useful in future research of branchial cofactors of AGD. The application of an effective probiotic community may prove helpful in generating a resilient microbiota of the gill surface, especially for the post seawater transfer phase. This in turn may mitigate against the proliferation of the detrimental pathobiome observed in chapters 3 and 4. It may also provide fish with greater resilience to AGD itself, by reducing potential pathogenic taxa development, and reducing the threat of a polymicrobial infection.

It would be crucial to apply the findings and outcomes of these studies to the field, where sampling of farmed Atlantic salmon from seapens may further contextualise the relationship to AGD outside of the *in vivo* laboratory environment. The farm environment contains a multitude of sources for environmental change and stress which are normally controlled within the laboratory environment, and these results would need to be validated in the context of AGD progression on farm. Further to this, development of a predictive modelling system for AGD based on microbiota profiles of the gill mucus or surrounding water may be possible to detect the early stages of the condition. Given the results obtained in chapter 4, groupings of AGD affected gill mucus and water from AGD-affected tanks were very strongly affiliated, and very distinct from AGD naïve counterparts. It has been shown that the presence of amoebiasis outbreaks can be predicted with a 79% accuracy rate based faecal microbiome samples (Morton et al., 2015), such is the correlation between *Entamoeba histolytica* presence and changes in the intestinal microbiota.

These areas of future investigation may provide further insight toward the pathogenesis of AGD, and the function and pathobiology of the causative agent, *N. perurans*. Furthered knowledge would simultaneously open new avenues for mitigation or treatment of this significant fish disease.

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Appendix 1 – Supplementary data for chapter 2

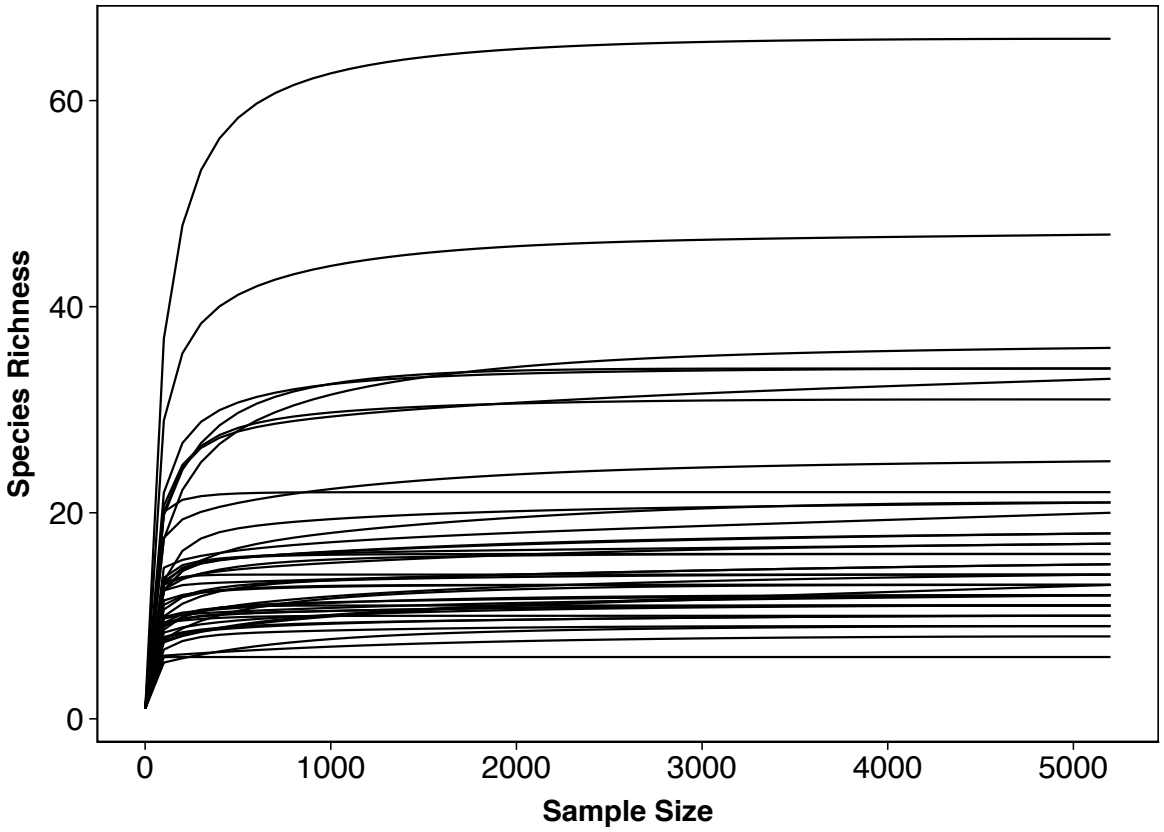

Figure S2.1. Alpha rarefaction curve depicting species richness (Observed ASVs) on the vertical axis, and sample size (expressed as sequence depth) on the horizontal axis. Good's coverage indicated >97% coverage for all samples at this sampling depth

Appendix 2 – Supplementary data for chapter 3

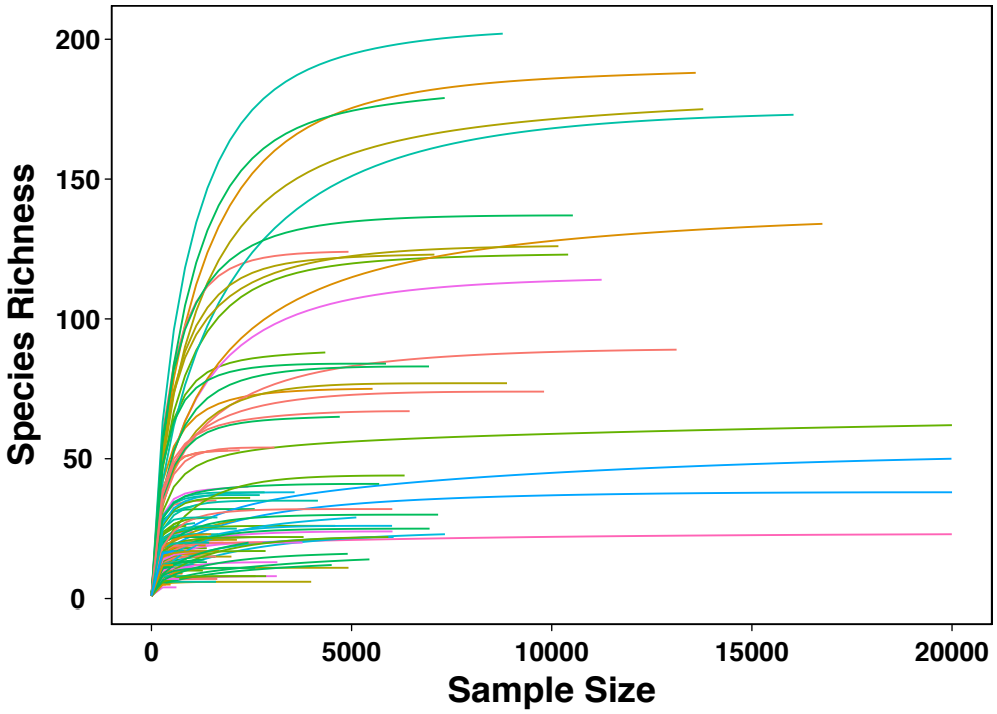

Figure S3.1. Rarefaction curve of gill mucus and water filter samples in the current study. Samples were subsetted to an even sampling depth of 7171, removing one failed H2O sample and one failed swab sample, along with the swab blank and NTC controls

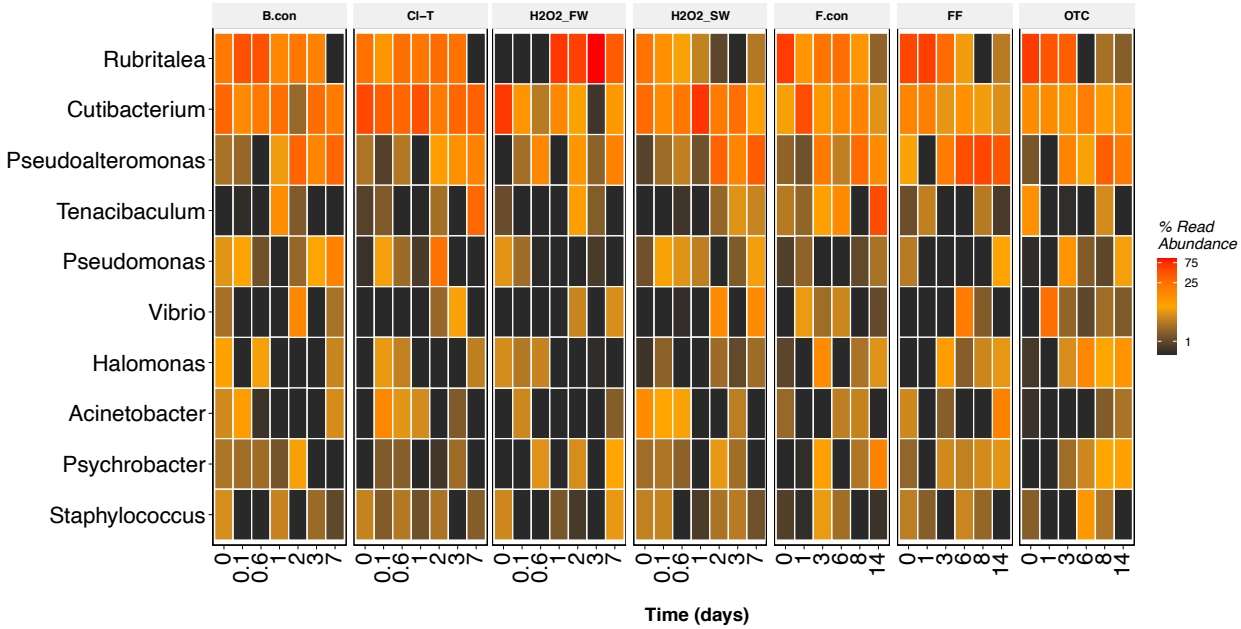

Figure S3.2. Heatmap of the 10 most abundant genus level assignments obtained from gill mucus samples, depicting relative abundance percentage of taxa temporally for each treatment group, for ease of visualisation of the three dominant taxon in this dataset.

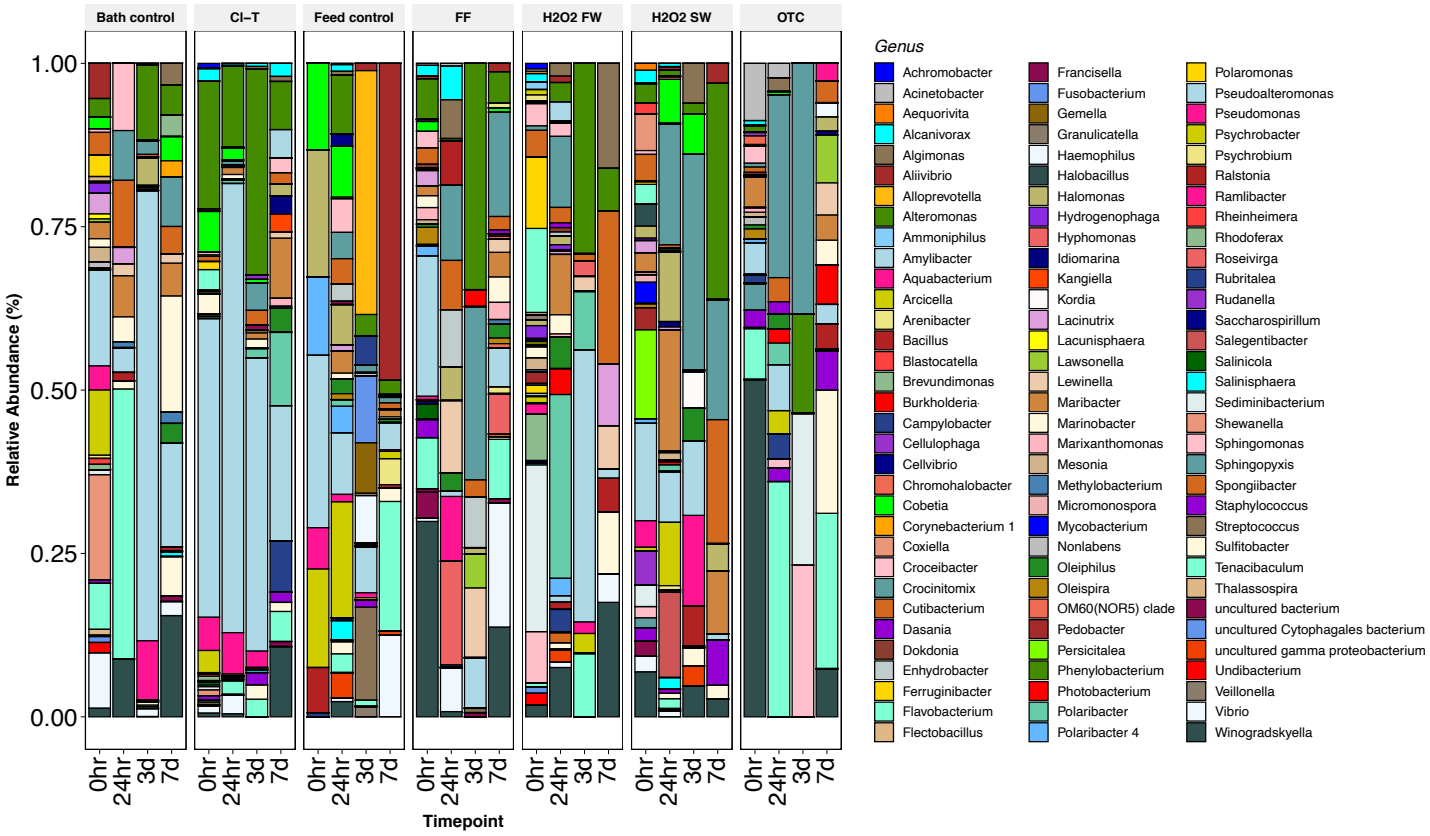

Figure S3.3. Relative abundance of genus level ASV assignments within tank water samples collected, displayed within treatment groups. Samples were collected at 4 timepoints throughout the trial (n=1 for each bar).

Appendix 3 – Supplementary data for chapter 4

Table S4.1. Primer sequence and concentration for qPCR assay target genes, *Neoparamoeba perurans* and salmon elongation factor 1 alpha (ELF)

Gene	Primer	Sequence (5'-3')	Reaction Conc. (nM)	Amplicon Length (bp)	Reference
N. perurans	Forward	AAAAGACCATGCGATTCGTAAAGT	300	70	(Downes et al., 2015, 2017)
	Reverse	CATTCTTTTCGGAGAGTGGAAATT	900		
	Probe	6FAM-ATCATGATTCACCATATGTT-MGB	200		
Ef1α	Forward	GGCCAGATCTCCCAGGGCTAT	900	66	(Bruno et al., 2007)
	Reverse	TGAACTTGCAGGCGATGTGA	900		
	Probe	NED-CCTGTGCTGGATTGCCATACTG-MGB	250		

Table S4.2. Primer sequences for the 2-step PCR preparation for amplifying the V1-V3 region within the 16S rRNA gene

Forward primer 5'-3' ("27F-adapt")		
Illumina overhang adapter	27F	Refs
TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG	AGAGTTTGATYMTGGCTCAG	(Lane et al., 1985; Zheng et al., 2015)
Reverse primer 5'-3' ("519R-adapt")		
Illumina overhang adapter	519R	Refs
GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG	GWATTACCGCGGCKGCTG	(Lane, 1991; O'Farrell et al., 2019)

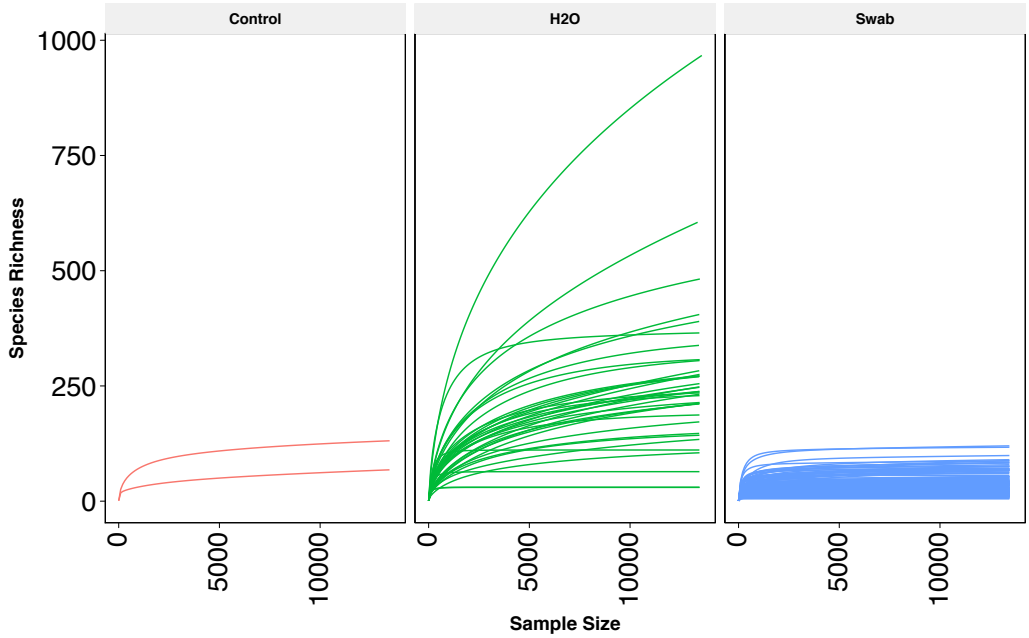

Figure S4.1. Alpha rarefaction curve from biological samples, depicting species richness (Observed ASVs) against sample size (sequence reads). Figure panels are faceted to sequencing controls, water samples and gill mucus swabs.

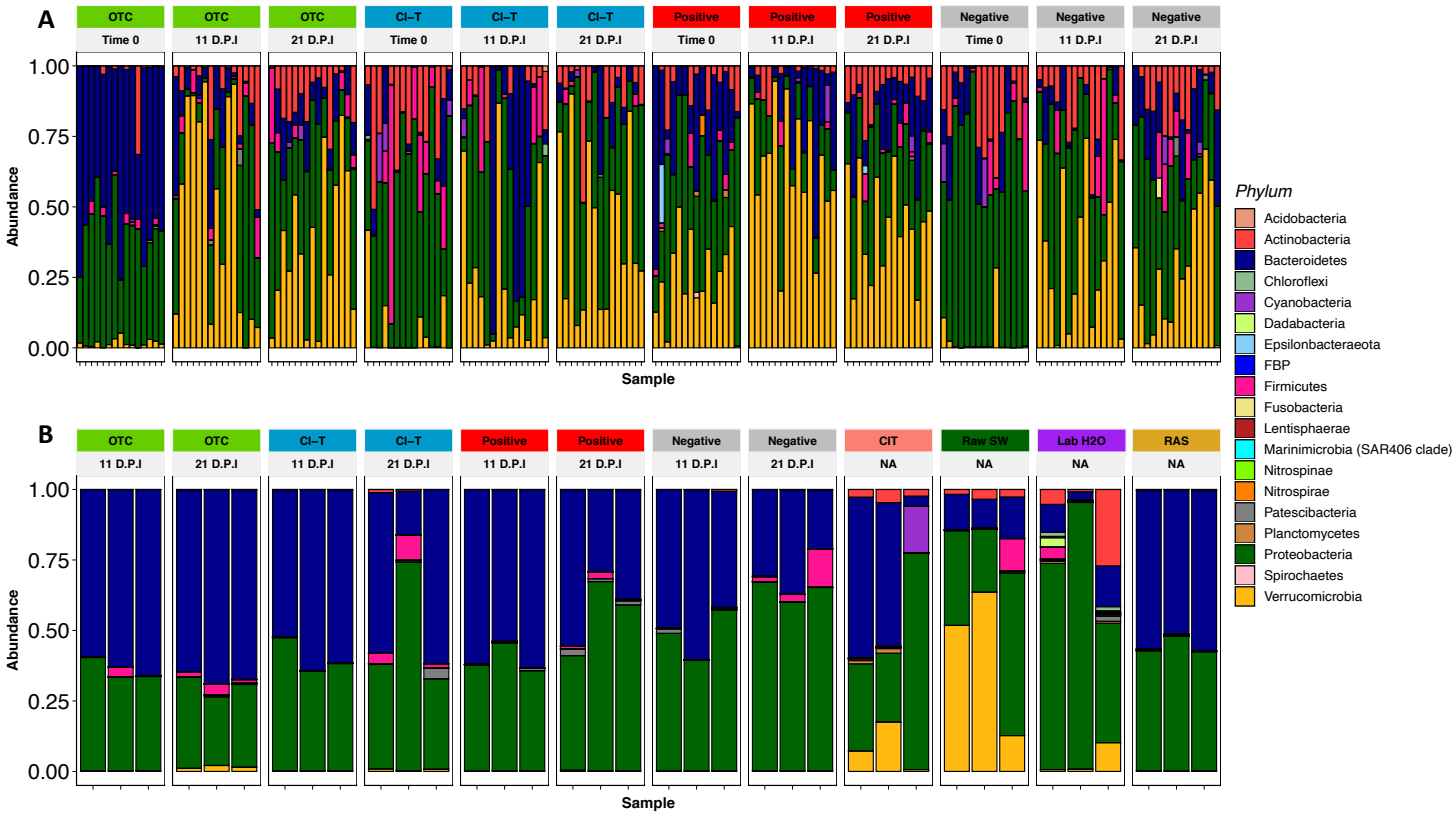

Figure S4.2. Relative abundance of phylum level ASV assignments for (A) fish gill mucus and (B) source water samples taken within the experimental trial. Data shows that fish gills were rapidly colonised by *Verrucomicrobia* post stocking in all treatments. Source lab water taken at the beginning of the trial also shared this ASV

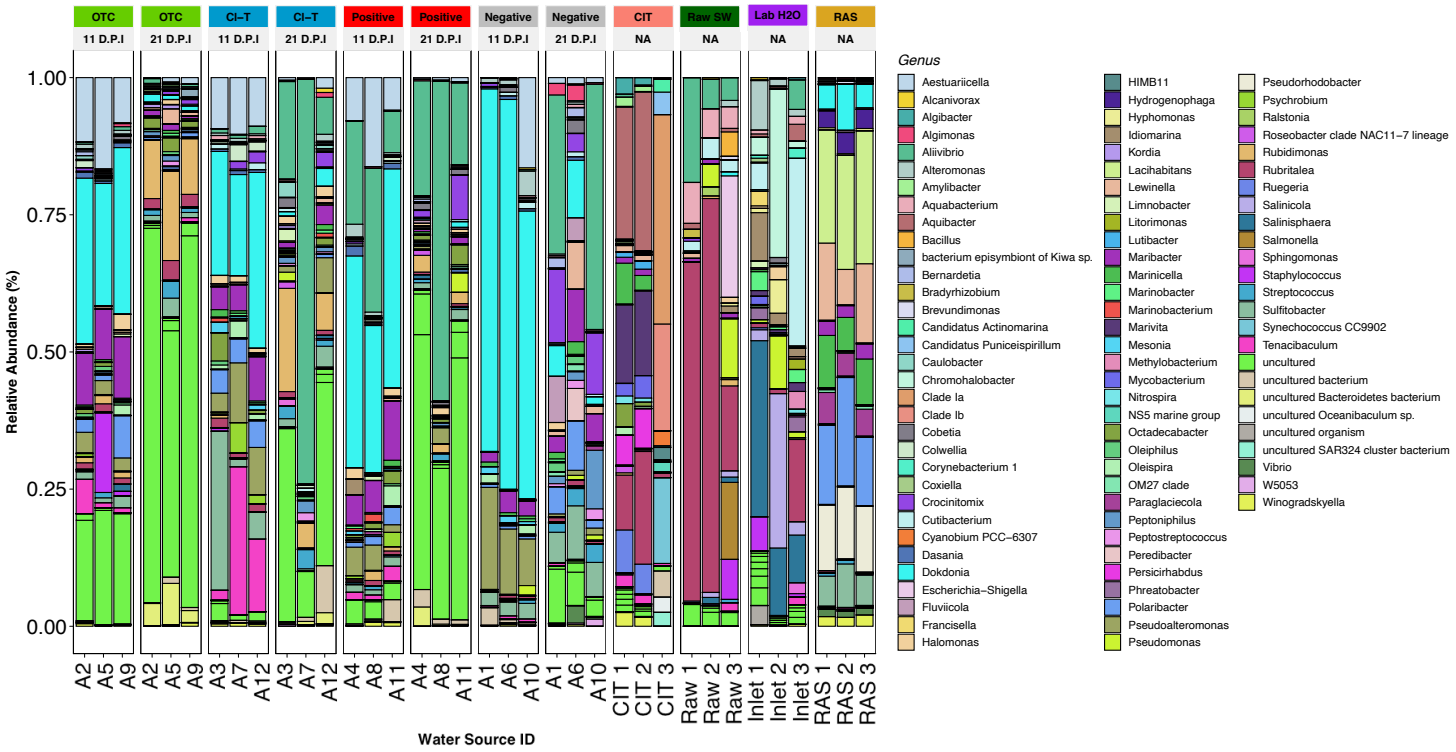

Figure S4.3. Top 91 genus assigned to various water sources used in the experimental trial, and tank water from experimental units. Each bar represents one pooled sample (3 x 700 ml water from each source). Data demonstrates that the assigned genus "uncultured" (Family *Saprospiracea* & *Caldilineaceae*) were prominent in all AGD affected treatments at 21 dpi.

Appendix 4 – Supplementary data for chapter 5

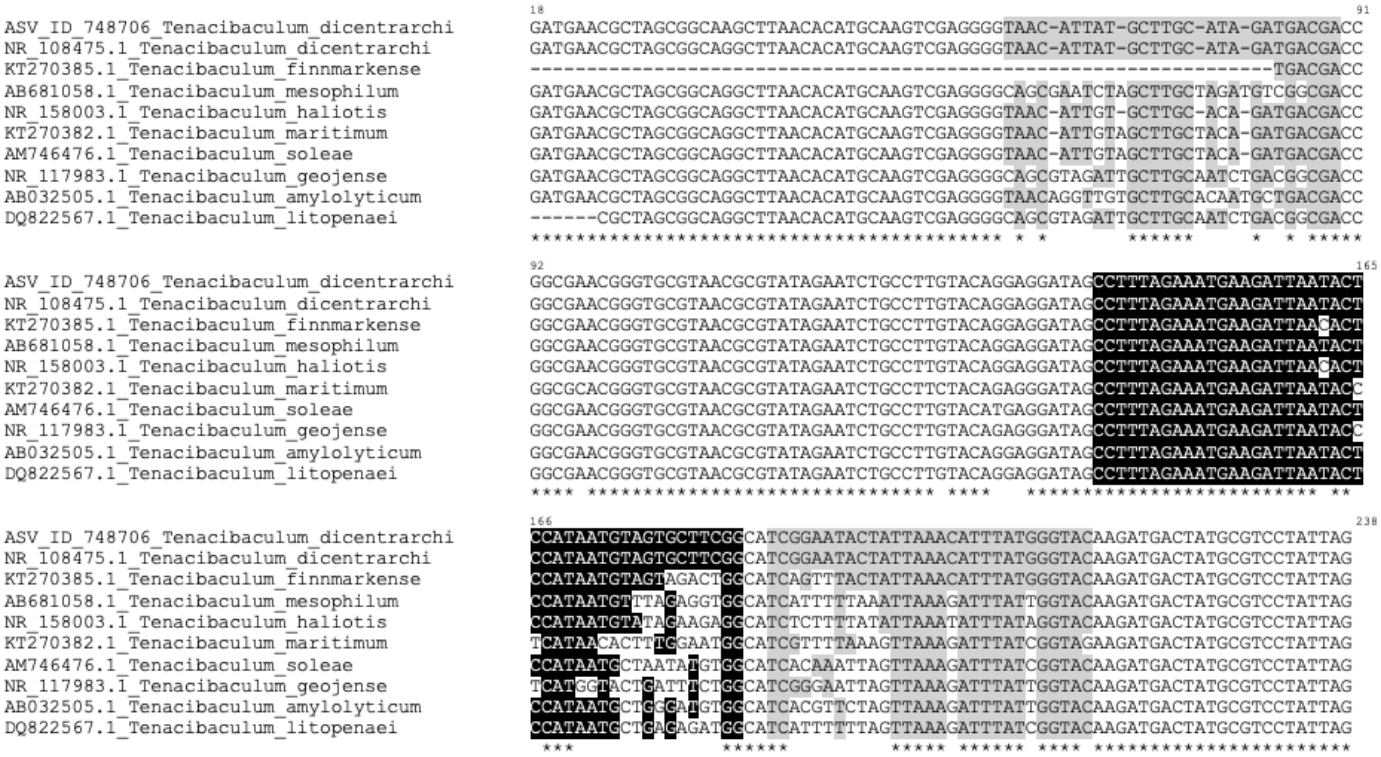

Figure S5.1. Sequence alignment of partial 16S rRNA genes from nine notable *Tenacibaculum* species (including accession number), and ASV sequence from the current study. The primer (grey shaded) and probe (black shaded) selections were based on unique conserved areas between the species sequences. Asterisk indicates no difference in nucleotide between species at each base position.

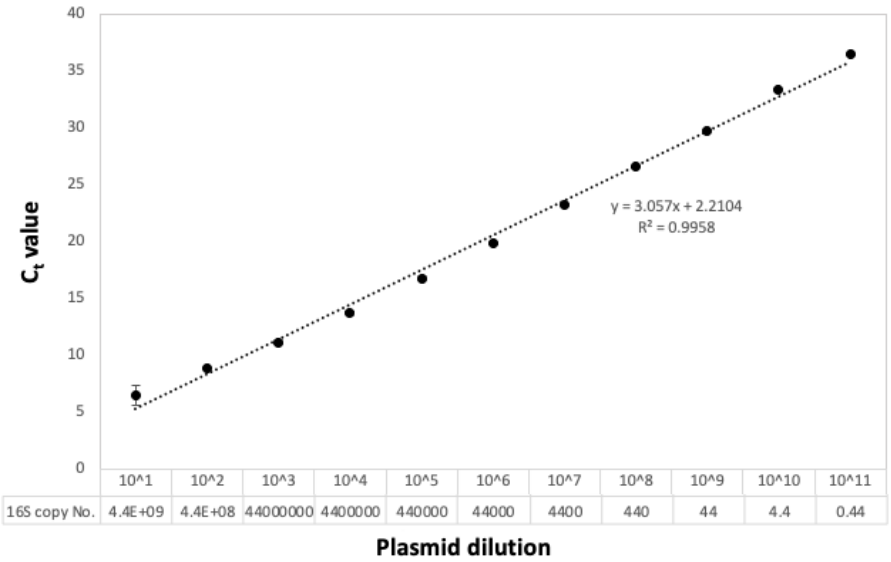

Figure S5.2. Standard curve obtained from 10-fold dilutions of *T. dicentrarchi* plasmid. At each dilution point the Ct value was plotted against dilution in triplicate.

Table S5.1. *Tenacibaculum dicentrarchi* quantitative PCR validation primer concentration. Combinations of primer concentrations were assessed using a fixed probe concentration of 100 nM.

	Forward and Reverse Primer Combinations (nM)								
	100F/100R	*50F/300R*	*50F/900R*	*300F/50R*	*300F/300R*	*400F/400R*	*300F/900R*	*900F/50R*	*900F/300R*
Mean Ct	**20.18**	20.08	20.00	20.58	20.11	20.24	20.11	20.65	20.92
SD	**0.03**	0.13	0.05	0.08	0.08	0.09	0.12	0.02	0.14

Table S5.2. Validation of probe concentration in *Tenacibaculum dicentrarchi* quantitative PCR assay. Various probe concentrations were assessed using a selected primer concentration of 300 nM (Forward) and 300 nM (Reverse).

	Taqman probe Concentrations (nM)					
	25nM	***50nM***	*75nM*	*100nM*	*125nM*	*150nM*
Mean Ct	20.04	**19.71**	19.76	20.20	19.95	19.62
SD	0.07	**0.08**	0.50	0.13	0.12	0.09
	175nM	*200nM*	*225nM*	*250nM*	*275nM*	*300nM*
Mean Ct	19.29	19.60	19.47	19.86	19.50	19.30
SD	0.28	0.14	0.43	0.23	0.33	0.14

Table S5.3. Limit of detection (LOD) for *T. dicentrarchi* qPCR assay.

***T. Dicentrarchi* LOD**					
16S Copy No.	*Ct 1*	*Ct 2*	*Ct 3*	*Mean*	*SD*
0.22	37.34	37.64	37.34	37.44	0.17
0.11	38.05	38.10	37.54	**37.90**	0.30
0.05	Und.	38.23	38.44	38.34	0.14
0.02	38.34	Und.	Und.	38.34	n/a