
GILL DISEASE IN FINFISH AQUACULTURE
WITH PARTICULAR EMPHASIS ON
AMOEBIC GILL DISEASE

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Submitted in fulfillment of the requirements of the degree of
Doctor of Philosophy

Supervised by Dr Eugene MacCarthy, Dr Ian O'Connor, Dr Hamish Rodger &
Dr Neil Ruane

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Title: Gill disease in finfish aquaculture with emphasis on amoebic gill disease


Abstract

Gill disease is one of the most significant challenges facing global salmon aquaculture and in terms of economic impact; amoebic gill disease (AGD) caused by the free living protozoan *Neoparamoeba perurans* is perhaps the most destructive. However, gill disease is often multifactorial, with numerous putative pathogens identified as potentially playing a role. AGD was first described in Irish aquaculture in 1995. Between the years 1995 and 2010, there were sporadic and relatively minor outbreaks of AGD. Since the re-emergence of the disease in 2011/2012, greater focus has been placed on gill health. This research aimed to investigate gill disease and in particular the re-emergence of AGD caused by *N. perurans* in Irish aquaculture. Through this it was hoped to provide the industry with the tools and information to help improve management of gill disease as well as fish health and welfare. With respect to this, Chapter 2 of this thesis details the effort to develop and validate a real-time TaqMan® PCR assay to detect *Neoparamoeba perurans* in Atlantic salmon gills. Furthermore, it describes the use of this assay to monitor disease progression on a marine Atlantic salmon farm in Ireland in conjunction with gross gill pathology and histopathology. As molecular diagnosis of AGD remains a high priority for much of the international salmon farming industry, Chapter 3 evaluates the suitability of currently available molecular assays in conjunction with the most appropriate non-destructive sampling methodology. In addition it compares this methodology with traditional screening methods of gill scoring and histopathology. Chapter 4 addresses the complex and multifactorial nature of gill disorders. Co-infections are common on farms and there is a lack of knowledge in relation to interactions and synergistic effects of these agents. The advances in molecular diagnostics have made it possible in Chapter 5 to identify *N. perurans* as the causative agent in the earliest AGD outbreaks. In addition to this, a number of other putative pathogens were also identified in these early cases of gill disease. Finally, Chapter 6 concludes the findings of this research and how they relate to the current knowledge of gill health and welfare.



Declaration

I hereby declare that the results presented are to the best of my knowledge correct, and that this thesis represents my own original work, carried out during the designated research project period, and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed:  _____, candidate

ID No. G00170432

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Chapter 1

Introduction

1.1 Salmon Aquaculture

Since the 1960s, successes in sea cage culture in Norway have led to the expansion and exponential growth of salmon farming globally. Atlantic salmon (*Salmo salar*) is one of the most intensively farmed marine fish and the main producers are Norway, Chile, Scotland, Canada, Australia and Ireland (Naylor & Burke 2005). Global salmon aquaculture production now exceeds 2 million tonnes per year, which has exceeded the wild harvest by over 1 million tonnes since 2004 (Fig. 1) (FAO 2017).

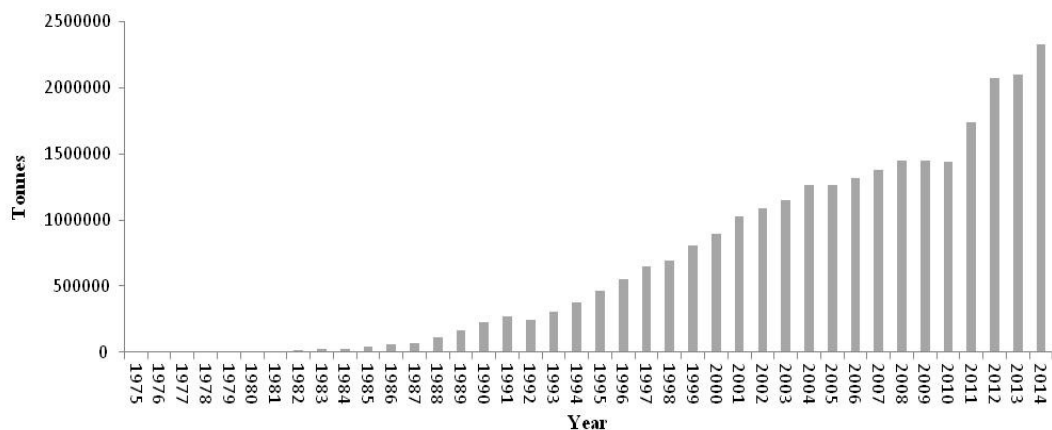


Figure 1. Global Atlantic salmon aquaculture production (Source; FAO 2017).

In Ireland, Atlantic salmon farming commenced in 1974 and produces c12,000 tonnes annually (Fig. 2). On a global scale the Irish industry is relatively small, < 1 per cent of global production, however, all production of Irish salmon is done to independently accredited organic standards, which are focused on low volume niche markets (Callier et al. 2011; BIM 2017). The number of active sites in Ireland varies from year to year due to management practices – in 2016 there were 22 sites in total in Ireland that contained salmon (O’Donohoe et al. 2017). Due to continued expansion and intensification, the emergence and recurrence of disease challenges is one of the major constraints on the sustainable development of the industry (Subasinghe et al. 2001). With respect to this development, greater attention is focused on the threat of parasites and their importance for aquaculture and the constraints posed to productivity (Scholz 1999).

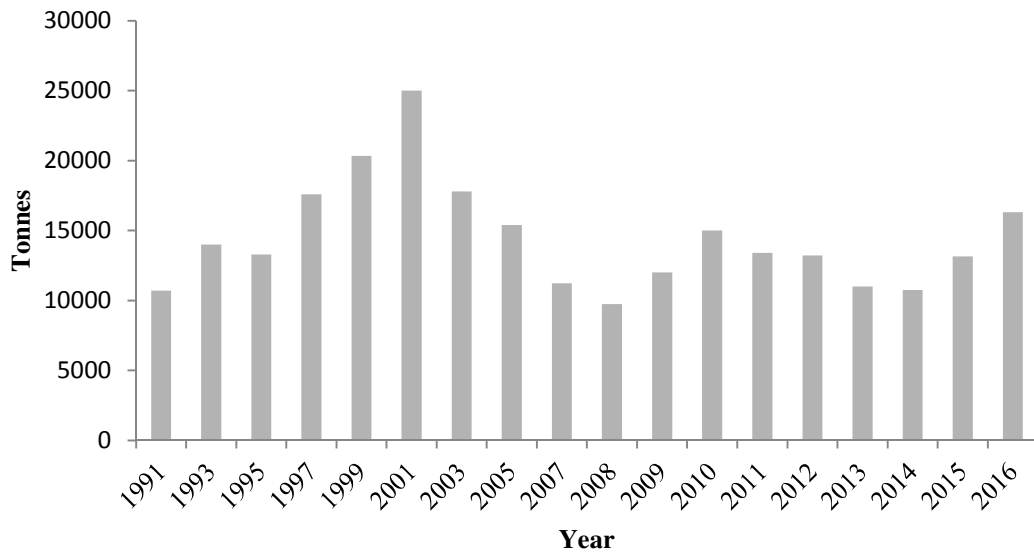


Figure 2. Annual Atlantic salmon production in Ireland between 1991 and 2016.

1.2 Gill Disease

The gill is a vital multifunctional organ that not only provides gas exchange, but also assists osmotic and ionic regulation and the excretion of nitrogenous wastes. Gill disorders pose a significant challenge to producers and are a cause of high levels of mortality in salmon (Rodger 2007). As they are in direct contact with the environment, gills are particularly susceptible to water-borne irritants, environmental changes and parasitic infections. Gill disorders are generally complex, often multifactorial and highly sporadic. There are several disorders that are attributed to an infectious aetiology such as Amoebic Gill Disease (AGD), Proliferative Gill Inflammation (PGI) and epitheliocystis (Table 1) (Mitchell & Rodger 2011).

However, the pathogenesis of a number of these agents is relatively unknown or questionable. Many of the gill disease cases or syndromes recorded appear to have a multifactorial aetiology (Mitchell & Rodger 2011), while non-infectious disorders due to harmful algae blooms (HABs) (phytoplankton and zooplankton) and other environmental challenges such as pollutants, nutritional or genetic deficiencies also play a role in mortalities attributable to gill diseases (Rodger et al. 2010). A significant cause of mortality in Ireland over the period from 2003 to 2005 was due to gill pathologies, which ranged from 1-79% (site dependent) and averaged around 12% (Rodger 2007). The actual casual agents of many of these pathologies had yet to be identified, but was believed to be a multifactorial condition involving environmental parameters, plankton in addition to a potential role of pathogens.

Table 1. Salmonid pathogens associated with gill disease in the marine environment.

| Pathogen | Group | Associated pathology/syndrome | |
|---|--------------|---|----------------------|
| <i>Neoparamoeba perurans</i> | Parasite | Amoebic gill disease | Young et al. 2007 |
| <i>Desmozoon lepeophtherii</i> | Parasite | Proliferative gill Inflammation (PGI) | Nylund et al. 2010 |
| <i>Trichodina sp.</i> | Parasite | Potentially destructive to gill structure | McArdle 1984 |
| <i>Gyrodactylus bychowskii</i> | Parasite | Obstructive gill damage | Bruno et al. 2001 |
| <i>Ichthyobodo spp</i> | Parasite | Marine Costiasis | Isaksen et al. 2012 |
| <i>Candidatus Branchimons cysticola</i> | Bacteria | Epitheliocystis/ PGI | Mitchell et al. 2013 |
| <i>Tenacibaculum maritimum</i> | Bacteria | Tenacibaculosis | Chen et al. 1995 |
| Salmon gill pox virus | Virus | PGI | Gjessing et al. 2015 |

1.3 Non-infectious Gill Disorders

Most notable of the non-infectious gill disorders associated with fish kills in sea cage culture are aggregations or blooms of gelatinous zooplankton. Such events are increasingly being reported in the literature, e.g. *Pelagia noctiluca*, which was implicated in a fish kill on a farm in Northern Ireland in 2007 (Purcell et al. 2007; Doyle et al. 2008; Delannoy et al. 2011). This species has since been intermittently observed in several other sites around Ireland (Marcos-López et al. 2014). Several species have been associated with mortality events, such as the siphonophores *Muggiaea atlantica* and *Apolemia uvaria*, hydromedusae *Solmaria corona* and *Phialella quadrata*, and the common jellyfish *Aurelia aurita* (Cronin et al. 2004; Ferguson et al. 2010; Baxter et al. 2011; Mitchell et al. 2011). Mortality due to zooplankton occurs through hypoxia, mechanical damage and the potential toxic effects when the nematocysts are discharged (Rodger et al. 2010; Baxter et al. 2011). There is also the potential for the jellyfish to act as vectors for bacterial infection, in particular secondary infections by *Tenacibaculum maritimum* (Ferguson et al. 2010).

The damage to both the shellfish and finfish aquaculture industry as a result of naturally occurring harmful algae blooms (HABs) is well documented, with between 60 and 80 species identified as toxic and a further 200 as having the potential to cause HABs (Smayda 1997; Rodger et al. 2010). Mortalities which occur due to HABs are caused by physical damage, asphyxiation due to oxygen depletion, oxygen super-saturation and ichthyotoxin (Black et al. 1991; Rodger et al. 2010). Eutrophication of coastal waters from anthropogenic sources, both terrestrial and aquatic, has been cited as a possible cause of the increased frequency and intensity of these blooms (Hallegraeff 1995). Several species of marine phytoplankton have been recorded as being associated with fish mortalities including *Karenia mikimotoi*, which is implicated in Atlantic salmon mortalities in Ireland, Scotland and Norway (Silke et al. 2005; Mitchell & Rodger 2007; Davidson et al. 2009; Rodger et al. 2010)

1.4 Amoebic Gill Disease

Amoebic gill disease is caused by the parasitic amoeba, *Neoparamoeba perurans*, which affects Atlantic salmon gills (Young et al. 2007). Previously, *Neoparamoeba pemaquidensis* was believed to have been the aetiology of AGD based on morphological (Kent et al. 1988; Dyková et al. 2000) and molecular characterisation (Wong et al. 2004). A mixed aetiology was proposed following the isolation of *Neoparamoeba branchiphila* from AGD-infected fish (Dyková et al. 2005). However, *N. perurans* was characterised and confirmed through Koch's postulates to be the agent of AGD (Young et al. 2007; Crosbie et al. 2012). The disease was first identified in the mid-1980s when it infected salmonids farmed in Washington state, USA and Tasmania, Australia (Kent et al. 1988). It has also been reported in South Africa, Chile, Canada, Norway, Scotland, Faroe Islands and Ireland (Rodger & McArdle 1996; Steinum et al. 2008; Bustos et al. 2011; Mouton et al. 2013).

AGD outbreaks are not just isolated to salmonids, with outbreaks observed in two separate cases from land based systems in ballan wrasse (*Labrus bergylta*) in Norway (Karlsbakk et al. 2013) and turbot (*Scophthalmus maximus*) in South Africa (Dyková & Novoa 2001; Mouton et al. 2013). The disease has also been recorded in ayu (*Plecoglossus altivelis*) (Crosbie et al. 2010) and blue warehou (*Seriolella brama*) (Adams et al. 2008). *Neoparamoeba* species have also been implicated in AGD in a farm containing olive flounder (*Paralichthys olivaceus*) in South Korea (Kim et al. 2005). Other species known to be prone to AGD are European seabass (*Dicentrarchus labrax*) and sharpsnout seabream, (*Diplodus puntazzo*) (Dyková & Novoa 2001; Dyková et al. 2005; Steinum et al. 2008). Left untreated, AGD can cause significant mortality, up to 10% of livestock per week (Munday et al. 2001), although freshwater baths are an effective treatment (Nowak 2012) for the disease. Additional costs are incurred through the reduction in fish growth rate and the removal and disposal of mortalities. The expense involved in freshwater treatments is due to the infrastructure and labour required, including the sourcing of large quantities of freshwater (Adams et al. 2012).

1.4.1 AGD in Ireland

The first case of AGD in Ireland was described in the autumn of 1995 in S1 Atlantic salmon transferred to sea in the spring of that year, with a total of 10 sites showing pathology and associated amoeba (Rodger & McArdle 1996; Palmer et al. 1997). Of ten sites with confirmed AGD, two recorded mortality exceeding 10%, while three others had less than 5% mortality, with the remaining sites experiencing no significant mortality (Rodger & McArdle 1996). Between the years 1995 and 2010, there were sporadic and relatively minor outbreaks of AGD (Fig. 3). Thought to be confined to warm dry summers, more widespread and sustained infections are now common (Rodger & McArdle 1996; Nowak et al. 2013, Rodger 2014) with approximately 50% of sites in Ireland affected in 2016. The peak phase in Ireland for the majority of new AGD outbreaks is June, July and August when sea temperatures are highest (Hamish Rodger, pers. comm.). The findings of the research in this thesis has also found that this period is most likely for new outbreaks of AGD in Ireland. Previous studies in Ireland demonstrated that although *Neoparamoeba* sp. were present on the gills of AGD affected fish, they did not necessarily correlate with the disease and a number of other amoebae species (*Platyamoeba* sp., *Nolandella* sp., *Mayorella* sp., *Vexillifera* sp.) were commonly found on the gills along with ciliate parasites (Bermingham and Mulcahy, 2006; 2007). However, it must be noted that these studies were conducted before the confirmation of *N. perurans* as the causative agent of AGD (Young et al., 2007; Crosbie et al., 2012) and without the use of species-specific molecular diagnostic tools. The occurrence of AGD in Ireland over the 2011/2012 period presented some unique challenges for the Irish salmon industry, in particular a shortage of well-boats for treating infected fish and permissions for the use of water sources by local authorities. Farms in Tasmania, which are located at sites with a strong influence of fresh water due to high levels of rainfall or located in a region with a strong freshwater input, are less impacted by AGD (Munday et al. 1993). However, AGD has also been observed in farms in Tasmania at temperatures of 10.6°C and salinity of 7.2 (Clark & Nowak 1999). During an AGD epizootic in Chile, rainfall was recorded lower than the 15 year average from May to November, and this was believed to be the most likely environmental factor for the timing of the outbreak

(Bustos et al. 2011). Outbreaks of AGD reported in Norway and Scotland were described as being associated with higher water temperatures (Steinum et al. 2008).

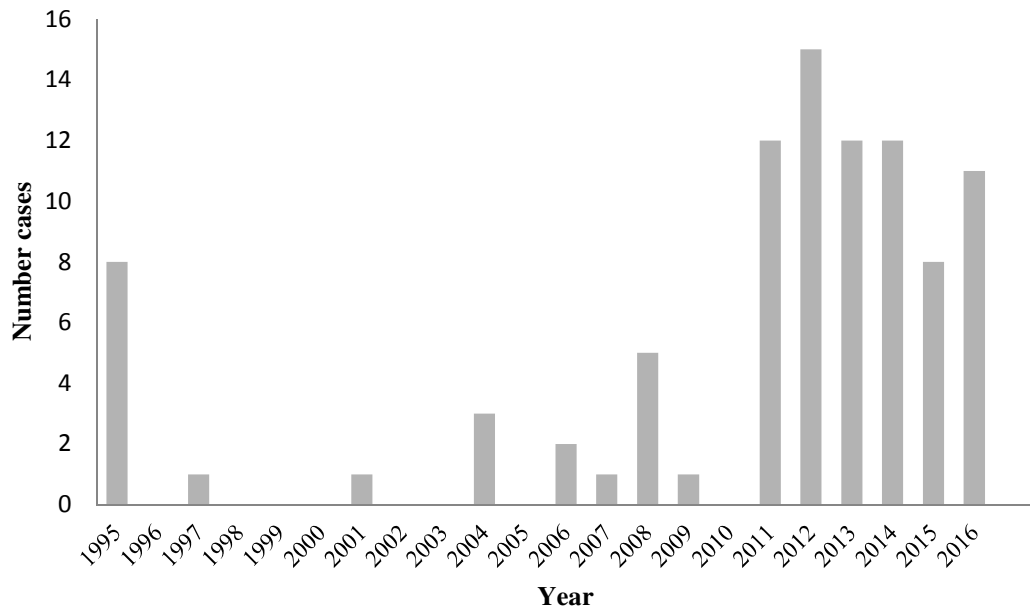


Figure 3. The number of confirmed new cases of AGD in Atlantic salmon between 1995 and 2016 in Ireland (Source: Fish Vet Group).

1.4.2 Pathology

The pathology of AGD has been well defined and is characterised by localised host tissue responses including epithelial oedema, hyperplasia of the epithelial cells as well as mucous cells, fusion of lamellae and the development of interlamellar vesicles (Clark & Nowak 1999). There may also be amoebae present in wet preps or observed attached in histological examination, which should contain at least one *Perkinsiella* amoebae-like organism (PLO) (Adams & Nowak 2004; Bustos et al. 2011) and this is considered to be case definition (Clark & Nowak 1999). Functional gill surface area can be severely reduced due to the filamental hyperplasia which causes inhibition in the exchange of carbon dioxide, leading to persistent respiratory distress (Powell et al. 2000). Further complications can arise as hypertension develops, causing circulatory collapse (Powell et al. 2002). Some cases in Scotland and Ireland have observed significant liver histopathology, which presents as multifocal necrosis (Rodger 2014). Importantly, the mechanism(s) by which *N. perurans* initiates the host response are not fully understood (Nowak et al. 2013). In transmission electron microscopy (TEM) analysis, enlarged swellings have

been observed in affected gill filaments with fusion of adjacent lamellae, in addition to spherical amoebae, which appeared to be embedded within the epithelium and which subsequently left indentations with visible fenestrations (Wiik-Nielsen et al. 2016). These fenestrated structures appeared to correspond with the presence of pseudopodia, which were observed in the study penetrating the epithelium.

1.4.3 Morphology and Phylogeny

The distinguishing feature separating *Paramoeba*, *Neoparamoeba* and *Janickina* from other species of amoeba is the presence of the endosymbionts or parasomes from the family Paramoebidae; an exception is the *Paramoeba eilhardi*, which sometimes lacked parasomes (Dyková et al. 2000; Kim et al. 2005). The genus *Paramoeba* is also distinguished by cell surface structure as it includes microscales or surface glycocalyx (Kim et al. 2005). The *Neoparamoeba* genus was established initially for *N. pemaquidensis* and *N. aesturina* as they lacked microscales, however they possessed a dense surface coverage of glycocalyx (Page 1987). When in motion, trophozoites of the genus *Paramoeba* and *Neoparamoeba* usually possess several dactylopodia as opposed to *Janickina*, which shows monopodial morphology (Kim et al. 2005). A comparative study completed by Dyková et al. (2005) acknowledged the importance of molecular characterisation, as differentiation between amoebae on a morphological level is almost impossible. The phylogeny of the amoebae associated with the Chilean epizootic was examined using the 18s rRNA gene and compared with the 18s rRNA gene sequences from 46 isolates of *Neoparamoeba* and a further out-group (Bustos et al. 2011). The phylogenetic analysis of the Chilean gene (GQ407108) sequence found that it clustered with the Australian and Norwegian isolates (EU326494) with 98.4-99.2 and 99.6% similarity respectively, which suggests that *N. perurans* has a universal distribution (Bustos et al. 2011). It has been suggested through phylogenetic analysis of *Paramoeba invadens* that it is most closely related to *Neoparamoeba* and *Paramoeba spp.*, and that on the nuclear SSU rDNA trees these two genera are phylogenetically inseparable, and therefore *Neoparamoeba* should be treated as a junior synonym of *Paramoeba* (Feehan et al. 2013). However, on the contrary, SSU rDNA is described as inadequate for separating the two genera and until further work has been completed on genes other than SSU rDNA, it is perhaps premature to

change nomenclature (Young et al. 2014). It was therefore decided to maintain the use of *Neoparamoeba* for the entirety of this study.

1.4.4 Diagnosis of AGD

Currently the most financially viable non-destructive means for the diagnosis of AGD on a commercial scale is through gross pathological assessment (Adams et al. 2004) using various gill scoring methods (Fig. 4) (Taylor et al. 2009). Gross pathological assessment and gill scoring methods have been utilised as a quantitative measure of the severity of amoebic gill disease in several studies and used as a monitoring tool on farms (Fig. 5). With the recurrence of AGD in Europe, gill scoring has quickly been adopted as the preferred method for monitoring of the disease. Development of the disease can be quite rapid, particularly in the summer months, with the majority of farms in Ireland performing gill checks on a weekly basis in conjunction with sea lice counts (Rodger 2014). Using tools such as gill scoring determines the severity of the AGD infection and the frequency of treatment (Nowak et al. 2013).



Figure 4. Atlantic salmon gill during gill scoring with established thickened mucus patches associated with AGD (Credit: Richard Taylor, CSIRO, Agriculture and Food).

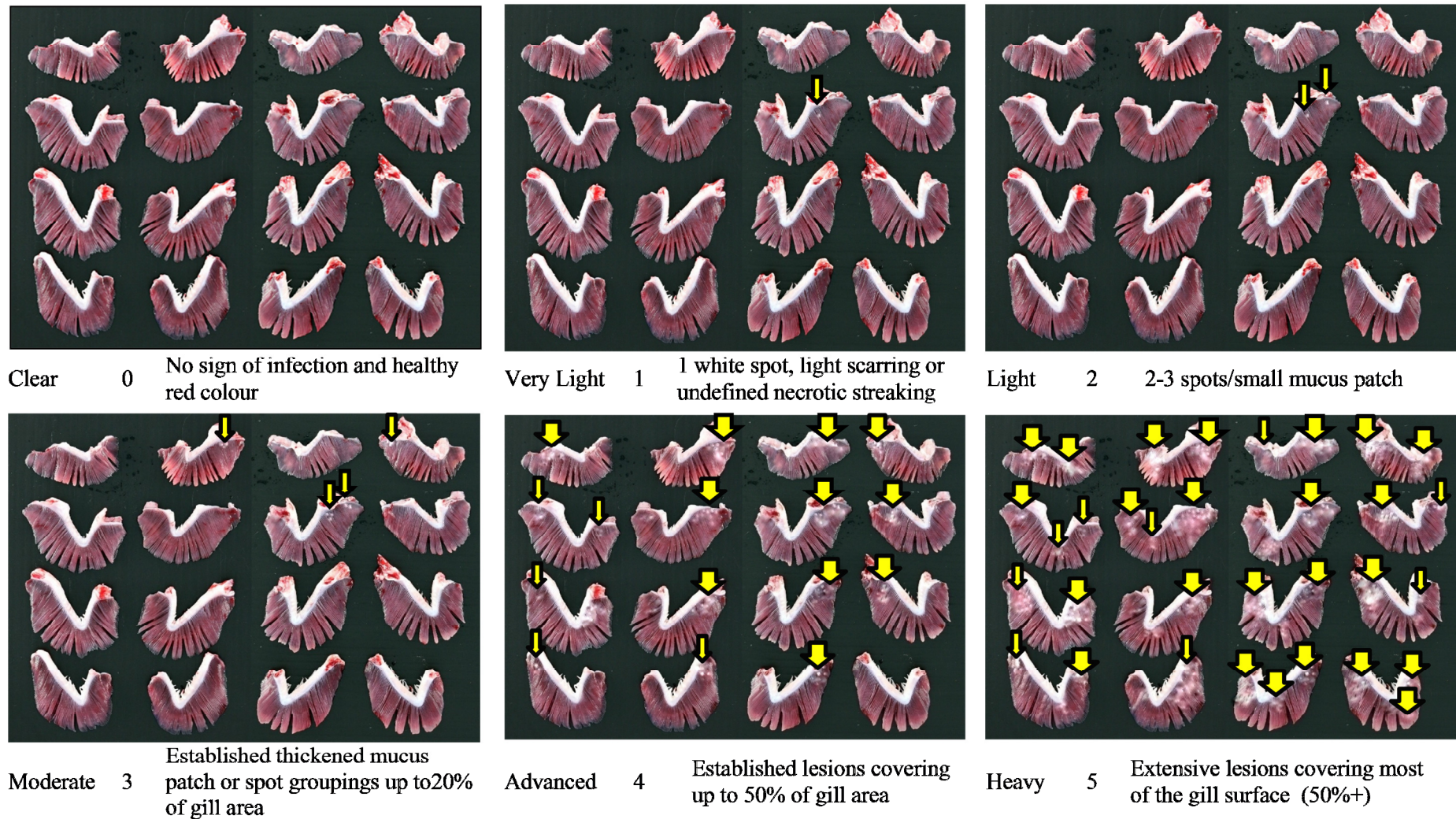


Figure 5. AGD Gill Score (0-5) from Taylor et al. (2009). Gill images show an illustrative development of AGD lesions across all 16 gill surfaces (Credit: Richard Taylor, CSIRO Agriculture and Food).

It is, however, a presumptive means by which to confirm the presence of AGD, and is open to misinterpretation. The detection of lesions and patches only indicates an altered gill condition but lacks the ability to identify the causative agent (Adams et al. 2004). As the reactions of gills are very few and look similar, lesions created by amoebae are difficult to distinguish from other pathogens or irritants, with the technique and experience of the observer also influencing the diagnosis (Adams et al. 2004). Lesions and patches on the gills do not always coincide with AGD in salmon and are less reliable in the early stages of an infection (Clark & Nowak 1999). Additionally, in species such as the lumpsucker, *Cyclopterus lumpus*, gill scoring is not practical due to a small operculum opening. The development of pathology is slower in lumpsuckers compared to salmon and they may act as carriers (Haugland et al. 2017). It has also been found that lesions and patches were reported to be absent in some locations and species (Palmer et al. 1997). These gross lesions, which are usually associated with AGD infection, are not necessarily present in infected turbot (Dyková & Novoa 2000). The severity of the lesions that are used to assess gill scores has been suggested to be related to the number of amoebae present on the gills, with the degree of amplification in the PCR analysis showing correlation with the level of infection (Bridle et al. 2010). Gross gill assessment is currently the primary means by which farms identify AGD and the severity of the disease. This method is dependent on a number of fish having relatively severe disease symptoms, which ensures that the disease is identified; however, as the disease progresses in severity some fish will inevitably die (Taylor et al. 2009).

While clinical diagnosis is accepted at farm level as a monitoring tool, further investigation through histological and/or molecular means is required for accurate diagnosis of the causal agent, particularly in new locations or species (Nowak et al. 2002). Histology has been one of the primary methods of identification and diagnosis of the causal agent, and it has also been utilised in the investigation of host response (Clark & Nowak 1999; Nowak et al. 2013). Mitchell et al. (2012) developed a histopathological gill scoring method, which assigned a score of 0 to 3 for each parameter associated with changes in gill health, including lamellar oedema, lamellar hyperplasia, lamellar fusion and circular anomalies (necrosis and sloughing). While both the gross and histological screenings have provided a valuable tool to the industry for the regulation of AGD, they are still limited in their capacity to identify the infectious agent (Young et al. 2008).

Currently histology is an invaluable tool in relation to the case definition of AGD, but it cannot illustrate all aspects of the host response – this is most clearly evident in artefactual loss of mucous and a certain portion of amoebae during tissue fixation and histology processing (Nowak et al. 2013).

A number of laboratory techniques were developed to confirm AGD in presumptively diagnosed fish, including immunofluorescent antibody test (IFAT) or immuno-dot blot, using polyclonal antisera raised against *N. pemaquidensis* (Douglas-Helders et al. 2001; Nowak et al. 2002; Young et al. 2008). Additionally a quick dip haematology stain was utilised on gill smears for rapid confirmation of AGD outbreaks on farms known to be affected by the disease (Zilberg et al. 1999). Other studies confirmed AGD by establishing cultures of the pathogens, which were then identified on the basis of morphology (Dyková et al. 2000). However, further analysis of morphological features suggested that this is not suitable for routine discrimination between *Neoparamoeba spp.*, and that PCR and phylogenetic analysis are more applicable (Wong et al. 2004).

In recent years, highly sensitive and species-specific methods of detection such as *in situ* hybridisation and PCR have become available since the discovery of *N. perurans* and are routinely performed in research and diagnostics. The recent development and use of these molecular methods has tended to focus primarily on *N. perurans*, which is surprising considering both *N. pemaquidensis* and *N. branchiphila* were previously considered the aetiological agent of AGD and have been isolated from the gills of AGD infected fish (Nowak et al. 2013).

Following the identification of *N. perurans*, Young et al. (2008) developed a PCR assay which amplified a 636bp region of the 18s rRNA gene (Table 2). Further investigation allowed for the development of *in situ*-hybridisation using oligonucleotides that bind with the 18s rRNA gene and this was utilised to confirm that *N. perurans* was the predominant aetiological agent of AGD in Tasmania, despite other amoebae species previously being associated with the disease (Young et al. 2008). The 18S rRNA gene is generally chosen due to its high copy number, which allows for high sensitivity, and is an established marker for microbial identification, with a database of species-specific sequences (Bridle et al. 2010). This assay was found to be specific and highly sensitive for the detection of *N. perurans* in gill samples and isolates of non-cultured gill-derived amoebae.

Bridle et al. (2010) developed and validated a real-time PCR assay using SYBR® Green chemistry and iQ5 Real-Time PCR detection system (Bio-Rad NSW, Australia). The primers used in this assay amplified a 146bp portion of the 18s rRNA gene from base 677 to 822 of *N. perurans* (Table 2) (Genbank accession number EF216903.1). A limit of detection (LOD) of 1.418 per reaction defined the lower limit and demonstrated an amplification efficiency of between 95 and 105%. However, no information in relation to melt curve analysis was provided in this study. Correlation between PCR results of gills swabs taken from infected salmon and gross gill scores showed potential for the development of a non-destructive sampling regime for the detection of AGD (Bridle et al. 2010). A quantitative duplex real-time TaqMan®-based PCR was developed for the detection of *N. perurans* in Atlantic salmon and rainbow trout, using a set of primers and probes to amplify a 139-bp fragment specific to the *N. perurans* 18s rRNA gene (Accession Number; EF216905.1) (Table 2) (Fringuelli et al. 2012). This assay was able to detect 13.4 DNA copies per µl of template and had an amplification efficiency of 104%, which is within the accepted level of amplification efficiency of 100+/- 10% (Purcell et al. 2011; Fringuelli et al. 2012).

Table 2. sequences for the current published methods for PCR and Real-Time PCR.

| Target | Primer/Probe | Sequence | Reference |
|--------------------|--------------|-------------------------------|------------------------|
| <i>N. perurans</i> | For | 5'ATCTTGACYGGTTCTTTTCGRGA3' | Young et al. 2008 |
| | Rev | 5'ATATGGTCTGCTTATCACTYATTCT3' | |
| | Peru For | 5'GTTCTTTTCGGGAGCTGGGAG3' | |
| <i>N. perurans</i> | Peru Rev | 5'GAACTATCGCCGGCACAAG3' | Fringuelli et al. 2012 |
| | Peru Probe | 6-FAM-CAATGCCATTCTTTTCGGA | |
| <i>N. perurans</i> | QNperF3 | 5'GTT TACATATTACCCACT3' | Bridle et al. 2010 |
| | QNperR3 | 5'TAA ACCCAATAGGTCTGC3' | |

Two types of chemistries, TaqMan® and SYBR® Green 1 dye, have been developed by Applied Biosystems for the detection of PCR products. Advantages and disadvantages for each type of chemistry are outlined by Applied Biosystems (Table 3). These differences are also outlined in the paper by Fringuelli et al. (2012), which identifies the importance of the reduced potential for TaqMan® chemistry to produce background or false signals due to the fluorescent signal being generated by the hybridisation of the probe to the target. This method also highlighted the ability

to detect and quantify in the same reaction both *N. perurans* and ELF (salmonid elongation factor-1 α) target genes, which reduced run-to-run variability. Although there are previous real-time PCR methods available based on both TaqMan® (Fringuelli et al. 2012) and SYBR® Green chemistry (Bridle et al. 2010), the ability of the former to detect *N. perurans* in field samples was not established. In particular, the incorporation of a minor-groove-binder probe (MGB) increases the melting temperature (T_m), allowing the use of shorter probes, thus providing greater specificity in comparison to the intercalating dye assays such as SYBR® Green.

Table 3. Advantages and disadvantages of the Sequence detection chemistries provided by Applied Biosystems.

| TaqMan® | | SYBR® Green | |
|---|--|--|--|
| Advantages | Disadvantages | Advantages | Disadvantages |
| <ul style="list-style-type: none"> • Specific hybridisation between probe and target is required to generate fluorescent signal • Allows for multiplexing • Post-PCR processing is eliminated, which reduces labour and material costs | <ul style="list-style-type: none"> • Synthesis of different probes is required for different sequences which can be expensive | <ul style="list-style-type: none"> • Can be used to monitor the amplification of any double-stranded DNA sequence • No probe is required | <ul style="list-style-type: none"> • May generate false positive signals as it can bind to any double-stranded DNA or bind to nonspecific double-stranded DNA sequences |

Treatments are triggered in general when moribund fish or fish with advanced clinical signs of disease are sampled. A diagnostic method which allows for the early identification of the aetiology agent is essential, particularly as the cost of treatments is highly demanding (Mitchell et al. 2012). Fish with no obvious pathology, either gross or histological, have previously tested positive via PCR when sampled using a gill swab, suggesting that once correctly optimised, a PCR assay could potentially be more sensitive than traditional diagnostic methods (Young et al. 2008).

1.4.5 Infection trials

For many years the advancement of research into the aetiology of AGD has been inhibited due to the inability to culture the causative agent (Crosbie et al. 2012; Nowak et al. 2013). Many difficulties in isolating and maintaining amoeba isolated from salmon gills have previously been highlighted as mixed populations of bacteria overgrow the amoeba (Dyková et al. 2000). Ciliates and flagellates, which originally colonised the gill, survived in agar plate culture, and early attempts to establish cultures in liquid media failed due to contamination of mixed bacterial overgrowth (Dyková et al. 2000).

Previously, studies attempting to elicit an AGD infection using different amoeba cultures were unsuccessful (Morrison et al. 2004). Atlantic salmon exposed to cultured gill-derived *N. branchilila* and *N. pemaquindensis* both failed to develop AGD (Morrison et al. 2005; Vincent et al. 2007). In the past, it was believed that an initial insult caused by the gills encountering a harmful water-borne agent was the initiation for an AGD infection or exacerbated the disease. However, a study by Adams et al. (2009) found that there was no significant difference between injured fish and control fish, suggesting that mechanical injury does not increase the risk of AGD. However, Crosbie et al. (2012) completed the isolation and the *in vitro* culture of *N. perurans*, and they were therefore able to fulfil Koch's postulates. The culture was maintained using malt yeast agar with seawater overlaid and subcultured every 3-4 days from which a clonal culture was established. After 70 days in culture a clone successfully infected Atlantic salmon causing AGD, which was subsequently re-isolated and confirmed by PCR and *in situ* hybridisation (Crosbie et al. 2012).

Clinical trials are the “*systematic studies in the species or in particular categories of the species for which a procedure is intended, in order to establish the procedure's prophylactic or therapeutic effect*” (Thrusfield 2013). Friedman et al. (2010) outlined four categories by which such procedures should occur:

- Pharmacological and toxicity trials, conducted to assess any potential negative effects that may be caused by the treatment product on the target species.
- Initial trials of therapeutic effect and safety, which are conducted on a small scale and usually in an environment where many variable

conditions can be controlled. Smaller trials such as these allow for the selection of the treatment product with the most beneficial outcomes.

- Clinical evaluation of efficacy. Following the establishment of the most beneficial treatment product, large-scale trials are conducted under operational conditions to assess management and environmental effects of the trial.
- Post-authorisation surveillance is carried out to monitor any adverse reactions that may arise.

In previous infection trials, AGD has been induced in the laboratory by cohabitation of naive Atlantic salmon with AGD-affected Atlantic salmon (Munday et al. 2001) or by scraping the gills of AGD-affected fish and placing the debris into fish-holding systems, which elicited AGD in naive Atlantic salmon (Zilberg et al. 2001). While these methods were consistent in initiating infections, variability in their severity had been noted as gill lesions appear to be proportional to the inoculating concentration or possibly due to the viability of the amoebae (Morrison et al. 2004). Concentrations of amoeba from 10 amoeba/L to 500 amoeba/L have been documented as causing AGD in naive Atlantic salmon, with pathology observed in both gross and histological examination appearing to be proportional to the concentration of amoeba used initially (Morrison et al. 2004). Differences in virulence between “wild” (amoebae extracted from AGD-infected fish) and cultured *in vitro* amoebae have been recorded throughout the research into AGD, and ideally, studies should be conducted using well-characterised strains of the *N. perurans*. Some evidence exists to show that cultures maintained in a lab for extended periods of time have displayed differences in virulence based on gill score (Collins et al. 2016). Furthermore, the clonal strain of *N. perurans* originally used to fulfil Koch’s postulates (Crosbie et al. 2012), was found to have lost virulence after 3 years in culture (Bridle et al. 2015).

As there is a significant cost associated with infection trials, most experiments have tended to focus mainly on the first infection after transfer to sea water and in particular most studies on immune response have been completed with naive smolts (Findlay et al. 1995; Vincent et al. 2006; Nowak et al. 2013). Future infections trials

could potentially include investigations into the immune response of fish that have experienced several re-infections.

1.4.6 Treatment of AGD

Although there has been significant research into treatments since AGD was first recorded, freshwater bathing remains one of the most effective and essential methods for the removal of the majority of amoebae that cause AGD (Parsons et al. 2001; Adams & Nowak 2004; Adams et al. 2012; Oldham et al. 2016). It was first documented in 1988 that a simple freshwater bath for 2-3 hours would provide immediate relief and recovery from AGD (Foster & Percival 1988). Current treatment strategies in Australia involve monitoring by gross gill lesions and prophylactic freshwater baths (Taylor et al. 2009). Re-infection of the gills can occur relatively quickly, varying from 1 to 2 weeks post freshwater bath, and increases in severity by 4 weeks (Clark et al. 2003; Adams & Nowak 2004). Since initial outbreaks in Australia in the 1980s, farms have seen a requirement for an increase in the frequency of treatments, with some fish being treated up to 15 times a year (Parsons et al. 2001; Rodger 2014). The mechanism by which freshwater bathing treats AGD is by osmotic effect, removing the excess mucus and the associated amoebae, thereby promoting healing of the gills (Clark et al. 2003). It was noted by Findlay et al. (2000) that fish placed in water with reduced salinity for an extended period of 4 weeks, allowing gills to fully recover, showed significant resistance to re-infection, which perhaps suggests the stimulation of adaptive immune response to AGD. Furthermore, Findlay et al. (2000) considered a number of factors, such as an interaction between immune responses, health of the fish and the gills, number of amoeba remaining following treatment, and environmental variables to be important in relation to the re-infection of AGD.

Treatments are generally triggered when farms observe 30 to 40% of fish with gill scores of 2 or above (Rodger 2014). While freshwater bathing is effective in significantly reducing amoeba gill load, with an 86+/-9.1% reduction in the amount of live amoebae observed, the remaining amoebae could potentially cause a re-infection within a week (Clark et al. 2003). It has also been observed that water hardness had a noticeable effect on the efficacy of freshwater bathing, with soft fresh water (19.3-37.4mgL⁻¹CaCO₃) proving to be more effective at reducing the numbers

of viable amoebae (73.9 to 40.9% of total count) (Roberts & Powell 2003). The physiological effects on salmon of freshwater bathing have also been investigated and it was found that freshwater bathing as a treatment posed very little risk of side effects (Powell et al. 2001). However, any form of bathing treatment can be problematic as it requires the fish to be confined by tarpaulin, cage skirt or transferred to a well-boat, which imposes a handling effect, causing acute stress to the fish (Powell et al. 2015).

During the emergence of AGD in Australia, research focused on establishing an alternative chemotherapeutic agent; however, much of this research was relatively unsuccessful. The results of these endeavours were summarised in a review of AGD research by Hardy-Smith & Humphrey (2011) and further reviewed by Oldham et al. (2016). Ten compounds demonstrated an inhibiting role in the growth of *Neoparamoeba sp.*, however, fewer again have been trialed to a commercial level. Levamisole is an immunostimulant which has been utilised in the treatment of nematode infections in both mammals and fish (Sakai 1999). In trials it has been found to have some inhibitory effects, but limited efficacy in a commercial setting (Findlay & Munday 2000). Treatments for pathogens, which infect sites that are not aided by a specific humoral immune response, such as mucous surfaces, prove difficult to treat and often require direct chemical therapeutics or changes/enhancement of the innate defence system (Findlay & Munday 2000).

A commonly used treatment in the aquaculture industry is hydrogen peroxide (H_2O_2), which is utilised in the treatment of many external parasites and gill infections as well as fungal, bacterial and protozoan infections, including sea lice infestations (Gaikowski et al. 1999; Schreier et al. 1996; Bruno & Raynard 1994). *In vitro* testing of hydrogen peroxide efficacy against *N. perurans* initially seemed to show promising results (Adams et al. 2012). Farms in Ireland and Scotland have good experience with using hydrogen peroxide for the treatment of sea lice, and had some success in treating cases of AGD in 2011 and 2012 at dosage levels between 1000 and 1400 mg/l for 18 to 22 minutes (Rodger 2014). However, a major disadvantage of hydrogen peroxide for the treatment of AGD is that there is a narrow safety margin and at temperatures $>13.5^{\circ}C$ its use becomes hazardous (Bruno & Raynard 1994; Rodger 2014). Mortality of 6.5-7.1% was recorded during *in vivo* trials with a concentration of 1250 mg L^{-1} at $12^{\circ}C$ and $18^{\circ}C$, which would be considered commercially unacceptable (Adams et al. 2012). The effects of hydrogen

peroxide on Atlantic salmon gills were investigated in relation to sea lice treatments and it was determined that exposure to 2.58gL^{-1} for 20 minutes causes complete mortality (Kierner & Black 1997).

The implementation of beneficial health management and husbandry practices has been highlighted as having the potential to reduce the impact and improve survival of fish infected with AGD (Nowak 2012). Included in such fish health management plans:

1. Reduced stock density
2. Net fouling/changing management
3. Mortality removal
4. Fallowing of site

In particular, fallowing of sites and cage rotation have been identified as having an effect on AGD, with fewer freshwater baths being required and increased growth rates observed where management practices were adjusted (Douglas-Helders et al. 2004). Novel strategies currently being investigated to mitigate against sea lice, such as snorkel cages, which encourage the fish to spend greater time at various depths (Frenzl et al. 2014, Stien et al. 2016), or light and feed manipulation (Bui et al. 2013), have also been suggested as alternative approaches for the management of AGD.

1.4.7 Transmission Pathways

It is widely accepted that disease interactions between feral and cultured fish occur regularly and one of the greatest challenges of marine parasitology is determining the environmental factors that are connected to the transfer of parasites between the wild and cultured animals (Scholz 1999; Mladineo et al. 2013). Amphizoic marine amoeba are believed to be ubiquitous in the environment, while reservoir populations, a mechanism of transmission to and among farmed fish for many disease causing amoebae, have not been fully elucidated (Nowak et al. 2010, Adams et al. 2012). Amoebae that can cause parasitic infections in farmed fish are known to be free-living in the environment and may alter their life strategies given the correct circumstances (Scholz 1999). Such infections may occur due to adverse impacts on the hosts from environmental stress factors, in particular elevated temperatures, salinity or initial insult from zooplankton, which can leave gills

susceptible to infection but are generally poorly understood (Nowak et al. 2013). Attempts to investigate environmental reservoirs of *N. perurans* in both the water column and sediment have been somewhat hindered as *N. perurans* has only recently been described as the correct agent of AGD (Nowak et al. 2010; Crosbie et al. 2012). Previous studies investigating potential reservoirs for the aetiology of AGD focused on *N. pemaquidensis* (Clark & Nowak 1999; Tan et al. 2002, Nowak et al. 2002). The combination of identifying the correct agent as well as the advent of molecular techniques has enabled further investigations of environmental reservoirs. Wright et al. (2015) detected *N. perurans* in the water column and determined that there were differences in the abundance of amoeba at different depths when amoeba numbers were highest.

A survey which screened 325 fish, including 12 different species collected in and around the surrounding area of salmon farms, was unable to find any fish infected with *Paramoeba* spp. (Douglas-Helders et al. 2002). *Paramoeba* spp. have been detected in the gills of wild cotta, *Thyrsites atun*, caught in the vicinity of Atlantic salmon farms (Foster & Percival 1988). An opportunistic sampling of a blue wahoo, *Serirolella brama*, from a cage containing salmon infected with AGD, was found to have a *Neoparamoeba* species present on the gills (Adams et al. 2008). Other species collected from the pens, a common jack mackerel, *Trachurus declivis*, and blue mackerel, *Scomber australicus*, were not infected. Much of this work was completed prior to the identification of *N. perurans* and before the establishment of sensitive PCR assays capable of detecting this species of amoeba. In Scotland, a survey of over 2,000 fish of various species collected from coastal waters found just a single individual, a horse mackerel, *Tachurus trachurus*, to be positive for *N. perurans* (Stagg et al. 2015). Most of the “wild” species of fish that have been found to be infected with AGD are species commonly found within sea cages. Mackerel, *Scomber scombrus*, lumpsuckers and wrasse (ballan & corkwing *Symphodus melops*) taken from infected cages in Ireland/Scotland have also been found to be infected with AGD (Hamish Rodger pers.comm.).

As greater emphasis is placed throughout the industry on the reduction of medicinal treatments for sea lice, a renewed interest in these cleaner fish as biological controls has emerged (Imsland et al. 2014, Powell et al. 2017). The identification of amoeba on the gills of cleaner fish species like lumpsuckers and wrasse is a major concern to the industry as these may act as potential reservoirs or

asymptomatic carriers (Haugland et al. 2017; Hellebø et al. 2017). This becomes more troublesome around treatments, as the current most effective treatment for AGD in Ireland is freshwater bathing, which is incompatible with the wrasse species. Lumpsuckers have appeared to show some tolerance to freshwater exposure (Powell et al. 2017), but further work is required.

There has been some evidence found to support the possibility of an association between *N. perurans* and *L. salmonis*, which may increase or prolong AGD outbreaks (Nowak et al. 2010). During an AGD epizootic in Chile, exceptionally high levels of co-infection with *Caligus rogercresseyi* may have contributed to the observed outbreak (Bustos et al. 2011). A heavy infestation of salmon lice may influence a case of AGD by increasing the burden on an already weakened fish or by contributing to the spread of the disease from fish to fish. A possible contributory factor to proliferative gill inflammation *Desmozoön lepeophtherii* (Mitchell & Rodger 2011) is known to infect the salmon louse (Freeman & Sommerville 2009), which highlights the potential of *L. salmonis* to act as a vector for AGD. During a preliminary survey of reservoirs for *N. perurans*, DNA of the amoeba was amplified from both alcoholic washing and whole animal extracts of *L. salmonis* which were collected from positive farms (Nowak et al. 2010). Further investigations into the potential for *L. salmonis* as a vector for AGD would be important, in order to fully assess if and how great a risk factor a heavy sea louse infestation would be.

The pathogenesis of re-infections in the post treatment period has been found to be identical to the initial infection, although a source of the re-infection was not identified (Adams & Nowak 2004). Potentially, the treated salmon themselves may be the main source of re-infection as some amoebae remain on the gills following treatment (Clark et al. 2003). Most studies that investigated the presence of such marine organisms in the environment were conducted prior to the description of the species (Tan et al. 2002; Crosbie et al. 2012) so this lack of knowledge in the area may warrant further investigation. Investigations by Nowak et al. (2010) were unable to detect the presence of *N. perurans* DNA in any sediment samples when in previous studies this was possible in relation to the species *N. pemaquidensis* and *N. branchiphila*, suggesting that there may be a difference in habitat or distribution of the species. However, with the subsequent identification of *N. perurans* and the development of sensitive real-time PCR assays, detectable populations of the

amoebae were found in high abundance from sites surrounding cage culture of Atlantic salmon, demonstrating that *N. perurans* is a free-living amoeba (Bridle et al. 2010). Further molecular surveys conducted in Norway found that only during clinical outbreaks were there detections of *N. perurans* in fauna, environmental samples and fish associated with salmon farms, suggesting that the greatest infection pressure is mainly from the AGD infected salmon themselves, emphasising the importance of early intervention (Hellebø et al. 2017).

1.5 Multifactorial Gill Disease

While AGD is perhaps the most significant disease in terms of gill health and economic impact (Steinum et al. 2008, Taylor et al. 2009, Nowak et al. 2013, Rodger 2014, Oldham et al. 2016), there are numerous putative pathogens that are potentially associated with gill disease (Mitchell & Rodger 2011, Gunnarsson et al. 2017). Proliferative gill inflammation (PGI) was the term introduced to describe recurring gill disease in Atlantic salmon in Norway with a multifactorial aetiology (Kvellestad et al. 2005; Steinum et al. 2010). This disease has been the cause of significant losses in Norway, with similar pathologies occurring in Scotland and Ireland (Mitchell & Rodger 2011; Matthews et al. 2013).

Bacteria such as *Tenacibaculum maritimum*, *Candidatus Piscichlamydia salmonis*, along with a number of other *Chlamydiae*, have previously been associated with gill disease. However, their role is still relatively unclear, particularly with respect to whether they are primary or secondary pathogens (Draghi et al. 2004; Mitchell & Rodger 2011; Ruane et al. 2013; Mitchell et al. 2013; Nylund et al. 2015). *T. maritimum* is the causative agent of tenacibaculosis (an ulcerative disease in marine fish, which is commonly known as eroded mouth syndrome), gill rot and gliding bacterial diseases (Mitchell & Rodger 2011; Fringuelli et al. 2012), with associated gill lesions being described first in chinook salmon *Oncorhynchus tshawytscha* (Chen et al. 1995). Gill infections due to tenacibaculosis tend to present with lethargic fish, causing an increased respiratory rate and increased mucus on the gills, along with pale and frank patches of necrosis (Rodger 2007). Preliminary diagnosis of symptomatic fish is carried out via microscopic examination of affected tissue showing motile filamentous bacteria (Mitchell & Rodger 2011). Further definitive confirmation should be carried out

through the isolation of bacterial colonies or using molecular diagnostics (Fringuelli et al. 2012). Transmission of the bacterium can be through seawater or directly from host to host, however, it has also been suggested that jellyfish may act as a vector for this pathogen (Ferguson et al. 2010; Delannoy et al. 2011) and therefore, *T. maritimum* may be responsible for secondary bacterial infections (Fringuelli et al. 2012; Ruane et al. 2013). Atlantic salmon have been found to be particularly susceptible to tenacibaculosis, with juvenile fish and temperatures above 15°C identified as risk factors (Soltani et al. 1996; Toranzo et al. 2005). It appears that in order to elicit clinical disease, some previous physical or toxic insult in addition to the increased temperature and poor husbandry are required (Mitchell & Rodger 2011). *T. maritimum* has also recently been isolated from lumpsuckers, *Cyclopterus lumpus*, which are increasingly used as a non-therapeutic means of sea lice (*Lepeophtheirus salmonis* & *Caligus elongatus*) control, giving added importance to the potential role of the bacteria in disease outbreaks in salmon culture (Smage et al. 2016).

Epitheliocystis is caused by intracellular Gram-negative bacteria described below, which affects the gills and skin of fish and has been reported in over 50 species, both marine and freshwater (Nowak & LaPatra 2006). Epitheliocystis is characterised by the development of inclusions/cysts in the brachial epithelium, in addition to the chloride cells (Paperna & Alves Dematos 1984; Bradley et al. 1988), as well as having been documented in skin epithelial cells (Hoffman et al. 1969). Pathology in gills associated with epitheliocystis includes hyperplasia, lamellar fusion and focal necrosis of epithelial cells (Draghi et al. 2004). Infections due to epitheliocystis have been described as being both proliferative and benign, depending on the agent and the host species (Bradley et al. 1988). In cases where there is proliferative epitheliocystis, fish have been described as lethargic and showing clear signs of respiratory distress (Mitchell & Rodger 2011). There are a number of bacterial agents associated with epitheliocystis in salmonids (Mitchell & Rodger 2011), however, recently *Ca. Branchiomonas cysticola* was identified as a potential agent of epitheliocystis in marine cultured Atlantic salmon (Toenshoff et al. 2012). A molecular study found that *Ca. B. cysticola* was found in far greater density in fish with large numbers of epitheliocysts in addition to *in situ* hybridisation identifying the agent within the cysts, which indicates a potential role for the agent in gill disease (Steinum et al. 2010). The significance of epitheliocystis

is continuously debated – some studies have indicated that the presence of the condition is merely coincidental (Clark & Nowak 1999), while others have observed it during PGI outbreaks with associated mortality (Kvellestad et al. 2005; Steinum et al. 2010). Gaps in knowledge relating to epitheliocystis have been identified, in particular its interactions with other gill pathogens, environmental factors and the pathogenicity of these organisms (Mitchell & Rodger 2011).

A microsporidian parasite, *Desmozoon lepeophtherii* (syn *Paranucleospora theridion*) has recently been described (Freeman & Sommerville 2009; Nylund et al. 2010). It is believed to have a complex life cycle involving both *L. salmonis* and Atlantic salmon (Nylund et al. 2010), although salmon have been found to be infected with the microsporidian in the absence of lice (Sveen et al. 2012). The true significance of this parasite as a gill pathogen is still unclear as it is frequently the most prevalent agent detected in gill samples, even in gills with no reported pathologies (Steinum et al. 2010; Mitchell & Rodger 2011, Gunnarsson et al. 2017). It has been suggested that *D. lepeophtherii* has a role in PGI. It is present in fish with PGI at up to 30 times greater levels than unaffected fish in one study (Steinum et al. 2010), with a 4 fold increase in another (Gunnarsson et al. 2017). In a case from Scotland it appeared that *D. lepeophtherii* was the causative agent of the gill disease outbreak recorded, which was associated with distinct proliferative and necrotic pathology (Matthews et al. 2013). It has been suggested that *D. lepeophtherii* may encourage immune suppression, thereby increasing the susceptibility of the host as well as facilitating the proliferation of pathogens already present in the fish (Magnadottir 2006; Nylund et al. 2010; Gunnarsson et al. 2017). Impaired immunity has been observed with another microsporidian, *Nucleospora salmonis*, which infects salmonids (Mitchell & Rodger 2011). Densities of the microsporidian appear to be influenced by environmental conditions, with higher densities being recorded during periods of highest temperatures (Sveen et al. 2012; Gunnarsson et al. 2017). It remains unclear whether environmental conditions are involved in triggering or augmenting the disease, and further work is required to fully characterise the relationship between the marine environment and potential gill disease pathogens (Matthews et al. 2013).

To date, two viruses (Atlantic salmon paramyxovirus (ASPV) and salmonid gill pox virus (SGPV)) have been identified as having some association with gill disease in Atlantic salmon, but their effect remains relatively unclear (Mitchell &

Rodger 2011). In 1995, a formerly undescribed virus belonging to the paramyxoviridae genus was isolated from the gills of Atlantic salmon suffering from PGI and was named ASPV (Kvellestad et al. 2003). However, subsequent infection trials failed to elicit pathology or mortalities in disease-free salmon, but the virus was associated with 2 cases of mortality in salmon farms in Norway (Fridell et al. 2004). Even so, further studies examining the multifactorial aetiology of PGI found no evidence for the involvement of ASPV (Steinum, Kvellestad, Colquhoun, Heum, Mohammad, Grøntvedt, et al. 2010). During a number of outbreaks of PGI in Norway, a DNA virus, SGPV, was first observed to infect epithelial cells causing hypertrophy and the degeneration of the nucleus, in addition to 20% and 80% mortality in freshwater and marine sites respectively (Nylund et al. 2008). During the outbreak in the marine site, *Neoparamoeba sp.* was also present, which may have contributed to the mortality (Steinum et al. 2008; Mitchell & Rodger 2011; Gjessing et al. 2015). The effect of SGPV appears to be greatest when recorded during freshwater production, and when it coincides with smoltification, significantly increased levels of mortalities have been recorded as the infection affects the gills and chloride cells in particular (Gjessing et al. 2017). With advances in molecular techniques, SGPV has been shown to be far more widely distributed than previously believed and is often found in addition to a number of other pathogenic agents (Gjessing et al. 2017), which further highlights the multifactorial nature of gill disease.

1.6 Objectives

The overarching goal of this doctoral thesis was to investigate gill disease, in particular the re-emergence of AGD caused by *N. perurans* in Irish aquaculture. To this end, the project initially set out with the specific aim of developing and optimising a sensitive and specific molecular diagnostic method for the detection of *N. perurans*. Furthermore, utilising this assay in conjunction with a longitudinal study enabled the research to establish the infection dynamics of AGD, which would support future management decisions. With the re-emergence of AGD in Europe there has been greater interest and effort focused on the disease; however, several different sampling methodologies and molecular assays have been employed. With a need to standardise sampling methodology across both research and industry, a

collaborative study with CSIRO, Australia was established to determine the most appropriate non-destructive molecular method to detect *N. perurans*.

While AGD caused by *N. perurans* is perhaps the most significant gill disease currently affecting salmon aquaculture, proliferative gill inflammation, which is thought to be caused by several potential agents, has also had a significant impact on fish health. Co-infections are not uncommon on farms and can potentially have a synergistic or antagonistic effect; however, co-infections have in general received limited scrutiny in the aquatic environment. A suite of molecular assays for putative gill pathogens was employed in order to try and determine the effects of co-infections during an AGD outbreak. Additionally, a retrospective study of the first recorded AGD outbreak was undertaken to gain a further understanding of the aetiology and epidemiology of gill disease. This work brings all these elements together in order to provide the industry with the tools and information required to ensure the sustainability of production and improve fish health and welfare.

This dissertation comprises 6 chapters, including an introduction and conclusion. The body of the thesis includes four chapters, of which two are peer-reviewed papers. The outline, objectives and publication details of each chapter are summarised below.

1.7 Summary of Chapters

Chapter 2: A longitudinal study of amoebic gill disease on a marine Atlantic salmon farm utilising a real-time PCR assay for the detection of *Neoparamoeba perurans*.

This study describes the development of an alternative TaqMan® assay for the detection of *N. perurans* according to MIQE guidelines and investigation of its application in monitoring the disease through a longitudinal study on a marine Atlantic salmon site during a single production cycle. Primary aims:

- Develop and fully optimise a TaqMan® assay for the detection of *N. perurans*.
- Investigate the re-emergence of AGD in Ireland through a longitudinal study.

This chapter has been published as a peer-reviewed publication:

Downes J, Henshilwood K, Collins E, Ryan A, O'Connor I, Rodger H, MacCarthy E, Ruane N (2015). A longitudinal study of amoebic gill disease on a marine Atlantic salmon farm utilising a real-time PCR assay for the detection of *Neoparamoeba perurans*. *Aquac. Environ. Interact.* 7, 239–251.

Presented to the following conference:

Downes J, Henshilwood K, Collins E, Ryan A, O'Connor I, Rodger H, MacCarthy E, Ruane N (2014). A longitudinal study of amoebic gill disease on a marine Atlantic salmon farm utilising a real-time PCR assay for the detection of *Neoparamoeba perurans*. *Aquac.* 2nd Annual Gill Health Initiative, Oslo, Norway, 21-23 May, 2014.

Chapter 3: Evaluation of non-destructive molecular diagnostics for the detection of *Neoparamoeba perurans*.

This study was conducted in conjunction with CSIRO, Australia in order to determine the most appropriate non-destructive sampling protocol for the detection of *N. perurans*. This compared two non-destructive methods of sampling, gill swabs and gill filament biopsy samples, which were tested with a range of currently available molecular assays to ascertain the optimal method for the detection of *N. perurans*. Primary aims:

- To assess a non-destructive sampling methodology, gill swabs and gill filament biopsy.
- Compare a range of currently available real-time PCR assays for the detection of *N. perurans*.
- Comparison of the non-destructive molecular diagnostics with traditional screening methods of gill scoring and histopathology.

This chapter has been published as a peer-reviewed publication:

Downes JK, Rigby ML, Taylor RS, Maynard BT, MacCarthy E, O'Connor I, Marcos-Lopez M, Rodger HD, Collins E, Ruane NM, Cook MT (2017) Evaluation of Non-destructive Molecular Diagnostics for the Detection of *Neoparamoeba perurans*. *Front Mar Sci* 4:61

Presented to the following conference:

Downes JK, Rigby ML, Taylor RS, Maynard BT, MacCarthy E, O'Connor I, Marcos-Lopez M, Rodger HD, Collins E, Ruane NM, Cook MT (2015) Evaluation of Non-destructive Molecular Diagnostics for the Detection of *Neoparamoeba perurans*, 3rd Annual Gill Health Initiative, Galway, Ireland 15-16 April 2015.

Downes JK, Rigby ML, Taylor RS, Maynard BT, MacCarthy E, O'Connor I, Marcos-Lopez M, Rodger HD, Collins E, Ruane NM, Cook MT (2015), Evaluation of Non-destructive Molecular Diagnostics for the Detection of *Neoparamoeba perurans*, 17th EAFP conference, Gran Canaria, Spain 7-11 September 2015.

Chapter 4: Investigation of co-infections with pathogens associated with gill disease in Atlantic salmon during an amoebic gill disease outbreak in Ireland.

Gill disorders can be complex and multifactorial with co-infections common on farms and there is a lack of knowledge in relation to interactions and synergistic effects of these agents. This study was undertaken to fully utilise valuable samples collected during a recorded outbreak of AGD over a full production cycle from 2013 to 2014 in Ireland using a suite of molecular assays in order to determine if and what effect a number of these agents may have on gill disease. Primary aims:

- Investigate the occurrences of a number of putative pathogens during an AGD outbreak.
- Determine whether there is any interaction between these agents or any potential synergistic effect.

Presented to the following conference:

Downes, JK., Yatabe, T., Marcos-Lopez, M., Rodger, H., MacCarthy, E., O'Connor, I., Collins, E., Ruane, N., (2017) Investigation of co-infections with pathogens associated with gill disease in Atlantic salmon during an amoebic gill disease outbreak in Ireland, 19th EAFP conference, Belfast, Northern Ireland. 4-8 September 2017.

Chapter 5: Confirmation of *Neoparamoeba perurans* on the gills of Atlantic salmon during the earliest outbreaks of amoebic gill disease in Ireland.

A retrospective molecular study was conducted on archived formalin-fixed paraffin-embedded (FFPE) Atlantic salmon gill material from samples associated with gill disease outbreaks and mortality events in 1995. Primary aims:

- Definitively identify the causative agent of the first recorded outbreak of AGD in Ireland.
- Additionally, investigate the presence of other putative gill pathogens in early gill disease outbreaks.

This Chapter has been submitted for review to the *Bulletin of European Association of Fish Pathologists*

Downes JK, Collins E, Morrissey T, Hickey C, O'Connor I, Rodger HD, MacCarthy E, Palmer R, Rutledge M, Ruane NM (2017) Confirmation of *Neoparamoeba perurans* on the gills of Atlantic salmon during the earliest outbreaks of amoebic gill disease in Ireland.

Chapter 6: Conclusions.

In the concluding chapter, the main findings are summarised and put into context for the Irish and international salmon aquaculture, with prospects for future research.

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Chapter 2:

A longitudinal study of amoebic gill disease on a marine Atlantic salmon, *Salmo salar* L., farm utilising a real-time PCR assay for the detection of *Neoparamoeba perurans*.

This chapter is a verbatim reproduction from the following published paper:

Downes J, Henshilwood K, Collins E, Ryan A, O'Connor I, Rodger H, MacCarthy E, Ruane N (2015) A longitudinal study of amoebic gill disease on a marine Atlantic salmon farm utilising a real-time PCR assay for the detection of *Neoparamoeba perurans*. *Aquaculture Environment Interactions* 7:239–251

2.1 Abstract

Amoebic gill disease (AGD) is a proliferative gill disease of marine cultured Atlantic salmon, with the free living protozoan *Neoparamoeba perurans* being the primary aetiological agent. The increased incidence of AGD in recent years has presented a significant challenge to the Atlantic salmon farming industry in Europe. In this study a real-time TaqMan® PCR assay was developed and validated to detect *Neoparamoeba perurans* in Atlantic salmon gills and further used to monitor disease progression on a marine Atlantic salmon farm in Ireland in conjunction with gross gill pathology and histopathology. The assay proved specific for *N. perurans* with no cross-reactivity with the related species *N. pemaquidensis*, *N. branchiphila* or *N. aestuarina*, capable of detecting 2.68 copies of *N. perurans* DNA. Although the parasite was detected throughout the marine phase of the production cycle, clinical AGD resulted in mortality peaks during the first twelve months only. The initial AGD outbreak resulted in peak mortality at week 17 which was preceded by PCR detections from week 13. Freshwater treatments proved an effective method for controlling the disease, resulting in a reduction in the weekly mortality levels and also a reduction in the number of PCR positive fish. In comparison to the more traditional diagnostic methods, the assay proved to be highly sensitive and a valuable tool for monitoring disease progression and has the potential to provide information on the timing and effectiveness of treatments.

KEY WORDS: Amoebic gill disease, *Neoparamoeba perurans*, diagnostics, Atlantic salmon

2.2 Introduction

Amoebic gill disease (AGD), caused by the parasitic amoeba *Neoparamoeba perurans*, is considered to constitute one of the major health challenges in marine cultured Atlantic salmon *Salmo salar*, (Young et al. 2007, Adams et al. 2012) and was first described affecting farmed salmonids in Tasmania, Australia and Washington state, USA in the mid-1980s (Kent et al. 1988). Over the last decade the disease has become more widespread and has now been reported in the majority of Atlantic salmon producing countries including Norway (Steinum et al. 2008), Chile (Bustos et al. 2011) and Scotland (Rodger 2014). In addition to salmonids, AGD has been reported in other fish species such as turbot *Scophthalmus maximus*, (Dyková et al. 1998, Mouton et al. 2013), sea bass *Dicentrarchus labrax*, sharp-snout sea bream *Diplodus puntazzo*, ayu *Plecoglossus altivelis* (Nowak et al. 2013), and also in ballan wrasse *Labrus bergylta* (Karlsbakk et al. 2013).

In Ireland, AGD was first recorded in 1995 on a number of marine Atlantic salmon sites (Rodger & McArdle 1996, Palmer et al. 1997) and continued to occur sporadically on a small number of sites since the first outbreaks (Bermingham & Mulcahy 2007). Initial outbreaks of the disease in Ireland were confined to warm dry summers, although in recent years more widespread and sustained infections have become more common (Rodger & McArdle 1996, Rodger 2014). If left untreated AGD can cause significant mortality of up to 10% per week, however freshwater baths of 2 to 4 hours have proven to be an effective treatment strategy (Munday et al. 2001, Parsons et al. 2001). An 86% reduction in the number of amoeba remaining in the gills has been observed following freshwater baths (Clark et al. 2003). However, this method of treatment can add extra costs, is labour intensive and several treatments may be required over the course of a production cycle (Nowak 2012).

Currently the most financially viable and non-destructive means for the assessment of AGD on a commercial scale is through the gross pathological assessment of the gill arches to identify multifocal lesions characterised by white mucoid patches (Clark & Nowak 1999, Adams et al. 2004) for which a gill scoring method has been developed (Taylor et al. 2009). However, this approach is a presumptive means by which to confirm the presence of AGD and is open to misinterpretation as the reactions of gills are limited and AGD-lesions are difficult to

distinguish from lesions caused by other pathogens or irritants. The technique and experience of the observer can also influence the outcome of the assessment (Adams et al. 2004). Therefore, the use of gill scores in the detection of lesions and patches only indicates an altered gill condition and does not specifically identify the aetiology (Adams et al. 2004). Lesions and patches do not always coincide with AGD in salmon and are less reliable in the early stages of an infection or less severe cases (Clark & Nowak 1999). While clinical screening is accepted at the farm level as a monitoring tool, further investigation through histological and molecular means is required for accurate identification of the causal agent, particularly in new locations or to identify different species of the genus *Neoparamoeba* (Nowak et al. 2002). Histological diagnosis of AGD is confirmed through observation of gill hyperplasia, lamellar fusion, vesicle formation and the presence of amoebae with an associated parasome (Clark & Nowak 1999, Rodger 2014). Histology is limited in its ability to specifically identify *Neoparamoeba* spp. as they are morphologically indistinguishable (Dyková et al. 2000). Both gross and histological examinations have been reported to underestimate the prevalence of AGD, particularly in the lower prevalence range (Clark & Nowak 1999).

In recent years, real-time PCR assays have become a more widely used diagnostic tool for the detection and identification of aquatic pathogens due to their robustness, sensitivity, high throughput and quick turnaround (Monis & Giglio 2006, Purcell et al. 2011). Since *N. perurans* was first described as the causative agent of AGD (Young et al. 2007) in marine-farmed Atlantic salmon, there have been two real-time PCR methods published, based on both SYBR® Green (Bridle et al. 2010) and TaqMan® chemistries (Fringuelli et al. 2012). TaqMan® chemistry is generally thought to offer several advantages over SYBR® Green (Martenot et al. 2010, Fringuelli et al. 2012). In particular, the incorporation of minor-groove-binders (MGB) that allow for the raising of melting temperatures of the probes (enabling the use of shorter probes) and the integration of the internal hydrolysis probe providing greater specificity in comparison to the intercalating dye assays due to the incorporation of any amplification products in the dye (Gunson et al. 2006, Purcell et al. 2011). In addition to this, the ability of the assay developed by Fringuelli et al. (2012) to detect *N. perurans* in field samples was not established and although the assay performed well, issues occurred with false negative results (defined as a negative PCR result from a fish sample with clinical AGD) in a number of field

samples tested by our laboratory (unpublished data). It was therefore decided to develop an alternative assay based on TaqMan® chemistry.

Molecular diagnostics have the potential to fulfil a role as an early warning and monitoring tool which would greatly compliment traditional diagnostic methods, particularly in the early stages of infection when clinical signs may be absent. The aim of this study was to develop an alternative TaqMan® assay for the detection of *N. perurans* according to MIQE guidelines (Bustin et al. 2009) and investigate its application in monitoring the disease through a longitudinal study on a marine Atlantic salmon site during a single production cycle. Results obtained from the molecular assay were also compared to gill scores and histopathology results, to determine if the assay could potentially provide a more rapid, sensitive and highly specific diagnostic tool in order to provide timely information on the initial infection and the potential timing of treatments.

2.3 Materials and Methods

2.3.1 Amoeba isolates and culture:

Neoparamoeba perurans was isolated from AGD affected farmed Atlantic salmon in the west of Ireland using a method adapted from Morrison et al. (2004). Gill samples from infected salmon were excised and placed into 25 cm² tissue culture flasks (Costar) filled with sterile seawater and transported, at ambient temperature, overnight to the laboratory. On arrival, culture flasks were screened for the presence of amoeba adhered to the flask surface. Once observed, the seawater was removed and the flask rinsed three times with sterile seawater. 0.5ml trypsin-EDTA 0.05 % (Gibco®) was added to the flask and monitored for 3-5 min until the majority of amoebae were free floating. A further 3 seawater washes were completed and the seawater transferred to a 50 ml falcon tube which was then centrifuged at 800 g for 10 min. The supernatant was discarded and the pellet re-suspended in 10 ml sterile seawater (salinity 35) and poured onto a 0.1 % malt yeast agar (MYA) plate. Amoebae cultures were maintained as described by Crosbie et al. (2012). *Neoparamoeba pemaquidensis* (ATCC®50172™) was obtained from the American Type Culture Collection (ATCC) and cultured according to the protocol provided. Additional ethanol fixed samples of *N. pemaquidensis* (strain

GILLNOR1/I), *N. branchiphila* (strain RP) and *N. aestuarina* (strain SU03) were kindly provided by the Institute of Parasitology, Academy of Sciences, Czech Republic.

2.3.2 DNA extraction and conventional PCR of cultured amoebae:

All DNA extractions (cultured amoebae, ethanol fixed amoebae and gill samples) were performed using a QIAamp DNA Mini kit (Qiagen) according to the manufacturer's instructions for animal tissue and the eluted DNA was stored at -20°C. To confirm the presence of *N. perurans* in the culture, amoebae were physically detached from the agar using a spreading bar and 10 ml of the amoeba-seawater solution overlay transferred to a 15 ml universal tube, which was immediately centrifuged at 800 g for 10 min. The supernatant was discarded and the amoeba pellet lysed in 180 µl of ATL buffer and 20 µl of proteinase K. Extracted DNA was tested by conventional PCR as described by Young et al. (2008). Additional DNA extractions were performed on *N. pemaquidensis*, *N. branchiphila* and *N. aestuarina* and universal eukaryotic primers (ERIB1 and ERIB10) selected from Barta et al. (1997) targeting the 18S ribosomal DNA gene were used for PCR amplification. All PCR products were run on a 1.5% (w/v) agarose gel in TAE buffer (40 mM Tris, 20 mM acetic acid, 2 mM EDTA), stained with ethidium bromide and visualised with the Quantity One, 1-D Analysis System software on a UV Transluminator (Bio-Rad). PCR products confirmed as *N. perurans* were subsequently purified and sequenced commercially (Sequiserve, Germany).

2.3.3 Real time primer and probe design:

The PCR primer pair and TaqMan® MGB probe were selected from alignments of previously published sequence data of the 18S rRNA gene sequences of *N. perurans* (EF216903-EF216905). Based on this alignment a forward primer "NP1" (5'- AAAAGACCATGCGATTTCGTAAAGT-3'), reverse primer "NP2" (5'- CATTCTTTTCGGAGAGTGGAAATT-3') and a probe "NPP" (6-FAM-ATCATGATTCACCATATGTT-MGB) were designed using Primer Express (Life Technologies). The primers generated an amplicon of 70bp and were obtained from Sigma; the probe was from Life Technologies.

2.3.4 TaqMan® real-time PCR:

Following assay pre-optimisation experiments (data not shown) using the Applied Biosystems standard protocols (<http://doc.appliedbiosystems.com>), each real-time PCR reaction mixture contained 5µl template, 12.5µl TaqMan® Universal 2x Master Mix (Applied Biosystems), 300 nM NP1, 900 nM NP2, 200 nM NPP and made up to 25 µl with MBG H₂O. The thermal profile of the real-time PCR program consisted of 15 min at 95°C, followed by 45 cycles of 15 sec at 95°C and 30 sec at 56°C in an Applied Biosystems AB7500 real-time instrument and associated software. Each run included a positive control, a negative control and a negative-process control (a blank sample extracted along with the gill samples). An internal process control (IPC; Life technologies) and external process control (salmonid elongation factor-1α; Bruno et al. 2007) were used for every 20 samples tested.

2.3.5 Validation of reaction efficiency, sensitivity and specificity:

Once confirmed as *N. perurans* the PCR product produced was then cloned into the pGEM® Easy Vector systems (Promega) and were purified using GenElute Plasmid Miniprep Kit (Sigma) according to the manufacturer's instructions. The plasmid concentration was measured spectrophotometrically at 260 nm and the value obtained was used to determine plasmid copy numbers, this was calculated using a DNA copy number calculator (<http://cels.uri.edu/gsc/cndna.html>). In order to determine the efficiency of the assay, Atlantic salmon gills from freshwater were spiked with *N. perurans* plasmid DNA and taken through the extraction process as described above. A 10-fold serial dilution was carried out in quadruplicate and each of the log dilutions were subjected to real-time amplification as previously described and only dilutions which provided C_t values in all replicates were used to generate a standard curve, created by plotting the C_t values against the 10-fold dilutions of *N. perurans*. Amplification efficiency of the real-time PCR assay was established based on the C_t slope method (Efficiency (Ex) = $[10^{(-1/\text{slope})}] - 1$) and the linearity was determined as the coefficient of correlation (R²). The dilution series was also used to determine the sensitivity of the assay. The lowest dilution, which provided C_t readings in all replicates, were investigated further via a 2-fold dilution series tested in quadruplicate, in order to determine the limit of detection. This final dilution was

then analysed a further 20x to assess the precision of the assay at a 95 % confidence level. The specificity of the assay primers and probe were initially determined theoretically using the Basic Alignment Search Tool (BLAST) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to identify potential cross reactivity with other species including Atlantic salmon. In addition, DNA extracted from *N. pemaquidensis*, *N. branchiphilia* and *N. aestuarina* were also tested using the real-time PCR assay.

2.3.6 Reproducibility:

The reproducibility of the assay was evaluated by screening seven different gill samples in triplicate. These samples had previously tested positive by both the Fringuelli et al. (2012) assay and the assay described in this manuscript. All samples were tested on three consecutive days and results were analysed in order to determine the coefficient of variation for intra-assay variation and also inter-assay variation i.e. the variation in each of the sample triplicates when compared between different PCR runs. The reproducibility was analysed by relative standard deviation.

2.3.7 Longitudinal study site and sampling details:

The longitudinal study was carried out on a marine Atlantic salmon fish farm on the south west coast of Ireland. The site is fully oceanic with little or no variation in salinity levels throughout the year. It is situated in an area that receives relatively high exposure, experiencing a mean wave height of 1.97 m and a maximum wave height of 8.28 m. The approximate depth of the bay where the site is situated is 23 m. 800,000 salmon smolts with an average weight of 60 g were transferred to the sea site during late April and early May 2013. Sampling commenced four weeks post transfer on the 3rd May 2013, when the average weight of the fish was 85 g, and continued until the 19th of September 2014 when the average weight of the fish was 4.6 kg.

At each sampling point, five feeding fish were selected from two fixed cages on site (n = 10) using a hand net. Moribund fish were avoided in order to ensure that fish sampled were representative of the population as a whole. At each sampling point gill scoring (0-5) was conducted on site using the method adapted from Taylor

et al. (2009) for AGD assessment. The second gill arch on the left-hand side was excised from each fish and immediately fixed in 10 % neutral buffered formalin for histological processing. Sections (5 µm) from paraffin embedded gill samples were stained with haematoxylin and eosin, and examined on an Olympus BX51 microscope. Based on the typical histopathology associated with AGD (fusion of the lamellae, hyperplasia, vesicle formation) and the presence of amoebae (Adams & Nowak 2001, Mitchell et al. 2012) a histopathology scoring scale was established for this study. The scoring system was based on Mitchell et al. (2012) and was applied to illustrate the progression and severity of the gill lesions in fish where AGD developed, a score of 0 = normal gill, 1 = low pathology (< 10% of gill filament affected); 2 = moderate pathology (< 50% of gill filament affected) and 3 = severe pathology (> 50% of gill filament affected).

The second gill arch on the right-hand side was excised from each fish and immediately placed in 1 mL RNA Later (Sigma) for molecular analysis. Total DNA was extracted from 25 mg of gill filament using the DNA Mini kit (Qiagen) and screened for *N. perurans* by real-time PCR as described above.

2.3.8 *In-situ* hybridisation:

Sections were hybridised with a digoxigenin (DIG)-labelled oligonucleotide probe specific to *N. perurans* as previously described (Young et al. 2008). Gill filament sections (7 µm) were placed on poly-L-lysine coated glass slides (Sigma), each section was deparaffinised in a series of xylene/ethanol washes. The proteinase K step was omitted. Sections were allowed to dry and a frame-seal (Biozym) was placed on the slides to make a chamber before overlaying with a mixture of 1 µl of DIG labelled probe (cultured amoebae DNA amplified using primers by Young et al. (2008) in 99 µl hybridisation buffer (5 ml formamide (Sigma), 1 g dextran sulphate (Sigma), 2 ml SSC buffer 20x (Roche), 2.5 mg tRNA (Roche), 200 µl Denhart's solution 50x (Sigma) and 2.8 ml dH₂O to a total volume of 10 ml). A cover slip was added and the DNA was denatured at 94°C for 5 min in a slide block (Bio-Rad Thermal cycler), then cooled directly on ice prior to overnight incubation at 42°C. The coverslips were removed and the slides were sequentially washed in 2x SSC buffer for 10 min (x2), 0.4x SSC buffer at 42°C for 10 min followed by 5 min in DIG1 buffer (0.10 M maleic acid, 0.15M NaCl). Each slide was then overlayed with

400µl of DIG2 buffer (1% blocking reagent (Roche) in DIG 1) and incubated in a humid box at room temperature for 30 min. Each section was given a short wash in DIG 1 buffer prior to an overlay of 400µl of DIG 2 buffer plus 1:500 anti-DIG-alkaline phosphate (Roche) and incubated for 1 hour in a humid box at room temperature. Slides were washed for 10 min in DIG1 (x2) and DIG 3 (0.1M Tris, 0.1M NaCl, and 0.05M MgCl₂H₂O) for 5 min. Each section was overlaid with 200µl of NBT/BCIP (Roche) in DIG 3 and incubated for 25 min. The colour reaction was stopped by 5 min incubation in DIG 4 (10mM Tris, 1mM EDTA) buffer followed by counter staining for 1 min with 0.5% Bismark Brown Y (Sigma) solution. Slides were then dehydrated in ethanol and xylene, before a coverslip was added.

2.3.9 Temperature and Farm data:

Temperature data was obtained using StowAway® Tidbit™ sensors which were attached to one cage pontoon at the site. Sensors were placed at depth of 10m and logged temperature on an hourly basis as part of the Marine Institute Temperature Monitoring Programme (www.marine.ie/home/publicationsdata/data/IMOS/IMOSTidbit.htm). Gill scores, mortalities and the number of freshwater bath treatments administered for AGD were recorded by the site manager. Mortality data was documented as the total weekly mortality per cage. Freshwater baths (2 – 3 h) were carried out at a number of time points during this study. These treatments were triggered based on the results of weekly gill checks, when farms observed 30 to 40% of fish with a score of 2 or above (Rodger 2014).

2.4 Results

2.4.1 DNA extraction and conventional PCR of cultured amoebae:

Neoparamoeba perurans was successfully isolated and cultured at 18°C on MYA plates, with washing occurring every three days and amoebae seeded onto fresh plates every two weeks. Cultured isolates (Fig. 6A) were tested via conventional PCR using *N. perurans* specific primers (Young et al. 2008) to confirm

their identity. Amoebae were also observed in fresh gill mucus scrapes from infected fish (Fig. 6B). Sequenced PCR products were analysed via BLAST and showed 99% similarity with sequences from Norway (KF146713), Australia (GU574794) and Chile (GQ407108). The 18S rDNA sequences retrieved from the ethanol fixed samples confirmed the identity of each amoeba species, following BLAST analysis.

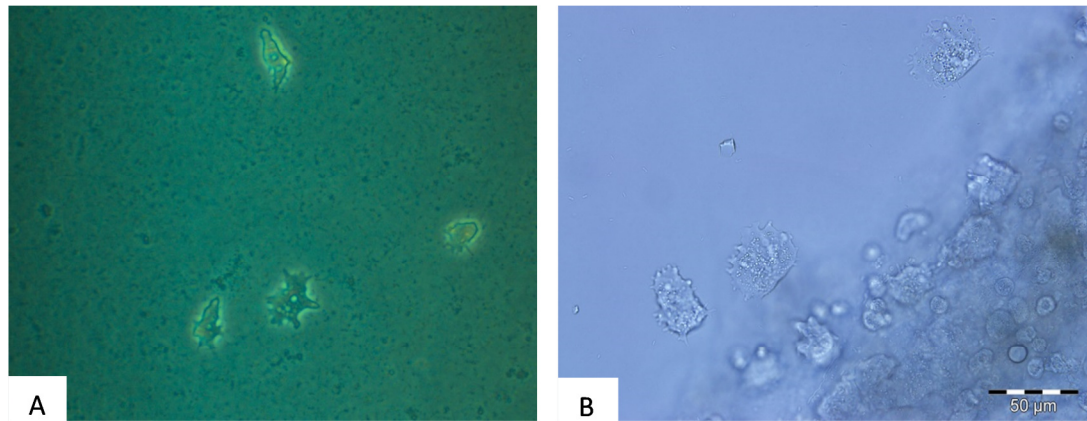


Figure 6. (A) *Neoparamoeba perurans* visualised growing on MYA plates in culture; (B) A fresh gill mucus scrape with amoeba migrating from the gills. Scale bar = 50 µm.

2.4.2 Real time primer and probe design:

Following assay pre-optimisation experiments, primer/probe final concentrations of 300nM NP1, 900nM NP2 primer and 200 nM NPP probe were used in all tests.

2.4.3 Validation of reaction efficiency, sensitivity and specificity:

The standard curve generated following testing of a 7-log dilution series of the amoeba plasmid spiked in Atlantic salmon gill had a slope of -3.363, an amplification efficiency of 98.44% with a linear correlation coefficient of $R^2 = 0.999$ (Fig. 7). The final dilution which produced a threshold cycle (C_t) value in all quadruplicates was 10^{-7} (Fig. 7). Analysis of serial dilutions (1:2, 1:3, 1:4, 1:5, 1:6 etc.) of the 10^{-7} dilution showed that consistent results were found in all replicates up to the 1:5 dilution giving the assay a reproducible cut-off C_t value of 40.13 (Table 4) equivalent to 2.68 DNA copies per μl^{-1} . This dilution was tested a further 20 times in duplicate to assure a 95% confidence (Table 5). Individual BLAST searches conducted on both the primers and probe sequences showed no similarity to any

other amoeba species. When tested experimentally with DNA isolated from *N. pemaquidensis*, *N. branchiphila* and *N. aestuarina*, no amplification was observed.

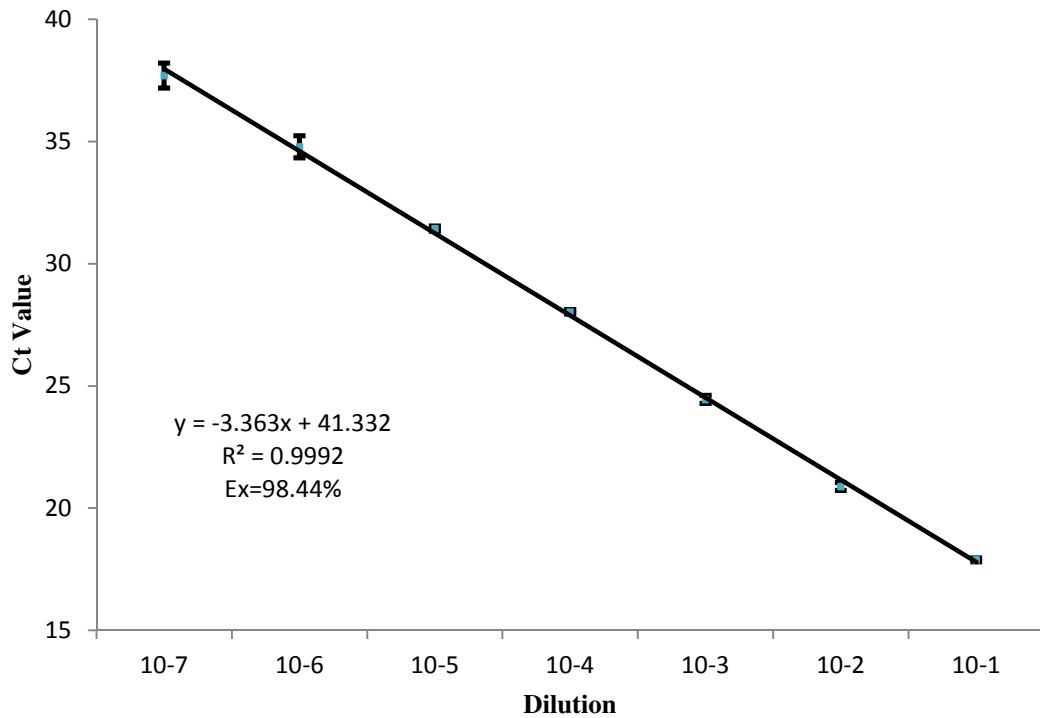


Figure 7. A standard curve derived from the amplification of quadruplicate log dilutions of *Neoparamoeba perurans* plasmid DNA in Atlantic salmon gill samples. At each point the Ct value was plotted against the dilution.

Table 4. Determination of the working limit of detection for the TaqMan® assay. The working limit of detection is indicated in the bold. nd: not determined.

| | Ct1 | Ct2 | Ct3 | Ct4 | Ct Mean | Ct Stdev |
|------------|------------|------------|------------|------------|----------------|-----------------|
| 1:2 | 37.71 | 37.43 | 36.64 | 36.00 | 36.95 | 0.78 |
| 1:3 | 38.03 | 39.72 | 38.90 | 37.42 | 38.52 | 1.01 |
| 1:4 | 40.12 | 38.66 | 38.96 | 39.24 | 39.25 | 0.63 |
| 1:5 | 40.31 | 40.02 | 40.36 | 39.81 | 40.13 | 0.26 |
| 1:6 | 42.83 | nd | 40.28 | 41.59 | 41.57 | 1.28 |

Table 5. The final dilution of the standard curve (Fig. 7) which produced Ct values in all replicates was further analysed and tested 20 times (in duplicate) to determine the precision of the assay at a 95% confidence level.

| | Ct value 1 | Ct value 2 | Ct Mean | Ct Stdev |
|-----------|-------------------|-------------------|----------------|-----------------|
| 1 | 37.48 | 38.81 | 38.15 | 0.94 |
| 2 | 38.96 | 38.07 | 38.52 | 0.63 |
| 3 | 39.57 | 40.44 | 40.01 | 0.62 |
| 4 | 39.54 | 39.24 | 39.39 | 0.21 |
| 5 | 39.32 | 38.51 | 38.92 | 0.57 |
| 6 | 38.67 | 39.44 | 39.06 | 0.54 |
| 7 | 39.5 | 39.22 | 39.36 | 0.20 |
| 8 | 38.75 | 39.33 | 39.04 | 0.41 |
| 9 | 40.47 | 40.94 | 40.71 | 0.33 |
| 10 | 39.53 | 38.47 | 39.00 | 0.75 |
| 11 | 39.49 | 40.26 | 39.88 | 0.54 |
| 12 | 39.25 | 39.21 | 39.23 | 0.03 |
| 13 | 39.51 | 40.14 | 39.83 | 0.45 |
| 14 | 39.57 | 39.7 | 39.64 | 0.09 |
| 15 | 39.82 | 39.39 | 39.61 | 0.30 |
| 16 | 40.39 | 39.45 | 39.92 | 0.66 |
| 17 | 40.58 | 39.94 | 40.26 | 0.45 |
| 18 | 39.85 | 39.01 | 39.43 | 0.59 |
| 19 | 38.99 | 39.36 | 39.18 | 0.26 |
| 20 | 40.15 | 39.55 | 39.85 | 0.42 |
| | | | 0.60 | 0.45 |

2.4.4 Reproducibility:

The mean intra-assay variances ranged from 0.05 to 0.62 % in the triplicates of the seven samples tested. Following the three separate repeats of the PCR assay, the inter-assay variation was found to range from 0.24 to 0.48 % (Table 6).

2.4.5 Longitudinal study – temperature, mortality and treatment dates:

Sea water temperatures, % weekly mortality rates and treatment dates for the entire production cycle are shown in Fig. 8. Sea temperatures ranged from 7.8 °C in April 2013 to 19.2 °C in July 2013. Two freshwater bath treatments were carried out on all cages in August (week 18) and September 2013 (week 24) with a further four treatments occurring on a number of pens on site in December 2013 (twice, weeks 34 and 37), and January (week 39) and June (week 61) 2014, respectively. Three periods of elevated mortality occurred during the production cycle resulting in peak weekly mortality rates of 2, 3 and 2.5 % respectively. However after week 38 (January 2014), weekly mortality rates remained below 0.5 %. The first increase in mortality, which was due to AGD, occurred on week 13, peaked at week 17 and declined from week 18 following a freshwater treatment. Mortalities due to AGD gradually increased again from week 22, but did not peak due to a treatment on week 24. A sharp increase in mortality occurred in week 26 due to a bloom of zooplankton, more specifically *Pelagia noctiluca*. The third period of high mortality, due to AGD, occurred between weeks 32 until 37 and resulted in a number of freshwater treatments for specific pens on site only (Fig. 8).

Table 6. Real time PCR Ct values from the reproducibility testing using 7 samples of cultured *Neoparamoeba perurans* tested in triplicate. The mean intra-assay variances ranged from 0.05 to 0.62% while the inter-assay variance was found to range from 0.24 to 0.48%. RSD-Relative Standard Deviation

| Sample | PCR Assay 1 | | | PCR Assay 2 | | | PCR Assay 3 | | | Inter-assay Variance | |
|----------|----------------------|------|------------|----------------------|------------|------|----------------------|------------|------|----------------------|------|
| | C _t Value | RSD% | Mean Ct±SD | C _t Value | Mean Ct±SD | RSD% | C _t Value | Mean Ct±SD | RSD% | Mean Ct±SD | RSD% |
| 1 | 33.50 | 0.13 | 33.50±0.05 | 33.43 | 33.40±0.07 | 0.20 | 33.49 | 33.55±0.07 | 0.19 | 33.48±0.09 | 0.26 |
| | 33.54 | | | 33.32 | | | 33.62 | | | | |
| | 33.45 | | | 33.44 | | | 33.55 | | | | |
| 2 | 32.55 | 0.31 | 32.60±0.1 | 32.42 | 32.51±0.08 | 0.23 | 32.45 | 32.44±0.05 | 0.16 | 32.52±0.10 | 0.31 |
| | 32.54 | | | 32.56 | | | 32.38 | | | | |
| | 32.72 | | | 32.54 | | | 32.48 | | | | |
| 3 | 32.57 | 0.20 | 32.63±0.07 | 32.42 | 32.52±0.10 | 0.29 | 32.63 | 32.62±0.02 | 0.07 | 32.59±0.08 | 0.24 |
| | 32.61 | | | 32.53 | | | 32.63 | | | | |
| | 32.70 | | | 32.61 | | | 32.59 | | | | |
| 4 | 31.91 | 0.05 | 31.93±0.02 | 31.55 | 31.76±0.20 | 0.62 | 31.68 | 31.76±0.08 | 0.24 | 31.82±0.13 | 0.42 |
| | 31.93 | | | 31.94 | | | 31.83 | | | | |
| | 31.94 | | | 31.8 | | | 31.78 | | | | |
| 5 | 37.25 | 0.51 | 37.26±0.19 | 36.95 | 37.14±0.17 | 0.46 | 37.00 | 36.98±0.03 | 0.08 | 37.13±0.18 | 0.48 |
| | 37.46 | | | 37.19 | | | 37.00 | | | | |
| | 37.08 | | | 37.28 | | | 36.95 | | | | |
| 6 | 34.32 | 0.60 | 34.46±0.21 | 34.36 | 34.46±0.08 | 0.24 | 34.43 | 34.56±0.11 | 0.32 | 34.49±0.13 | 0.39 |
| | 34.70 | | | 34.51 | | | 34.60 | | | | |
| | 34.37 | | | 34.50 | | | 34.64 | | | | |
| 7 | 33.41 | 0.47 | 33.59±0.16 | 33.5 | 33.61±0.11 | 0.32 | 33.39 | 33.43±0.09 | 0.26 | 33.55±0.14 | 0.41 |
| | 33.68 | | | 33.71 | | | 33.53 | | | | |
| | 33.69 | | | 33.63 | | | 33.37 | | | | |

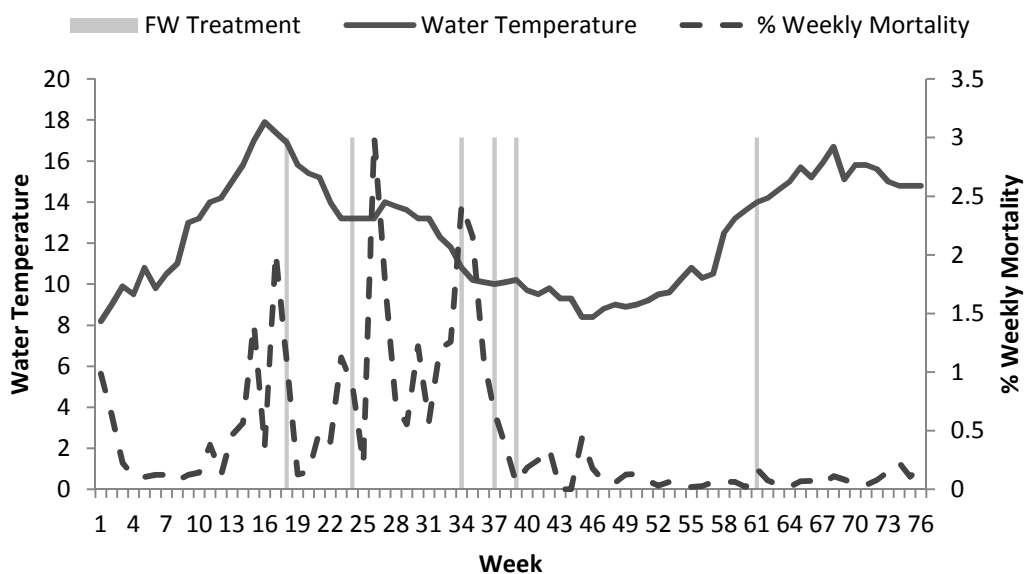


Figure 8. Seawater temperatures, % weekly mortality rates of Atlantic salmon and the dates of freshwater bath treatments throughout the marine production cycle during the longitudinal study.

2.4.6 Longitudinal study – PCR results, gill scores, histological scores

Sampling was initiated on week 4 (3rd May 2013) and completed on week 76 (19th September 2014) before harvesting began. The average gill score, average histological score and % PCR positive results for the entire production cycle are shown in Table 7. The first PCR positive samples were detected in week 4, however, all fish sampled in week 6, 8 and 9 were negative (Table 7, Fig. 9A). The numbers of PCR positive fish started to increase from week 12 until week 16 when all fish were positive and coincided with the first peak of mortality. Following the freshwater treatment on week 18, only 10 % of fish were PCR positive on week 19, increasing to 80 % by week 24 due to the second AGD outbreak. Following the second full site treatment, 30 % of fish were positive on week 28 before increasing again to 100 % by week 32, prior to the third AGD outbreak on site. During the second half of the production cycle, PCR positive fish were detected up to the pre-harvest period on week 76. The first increase in mean gill score was observed in week 16 (31st July 2013), coinciding with the first outbreak of AGD with average gill score of 2.5 (Fig. 9B). Following treatment, the mean gill score declined to < 0.5 by week 21 before increasing again (to 1.5) on week 24 after the second AGD outbreak. During the third AGD outbreak and for the remainder of the production

cycle, moderate mean gill scores were observed but were never greater than 2. The average histological gill score first increased (to 2.5) on week 16 (Fig. 9C) and gradually reduced following treatment showing a similar pattern to the mean gill scores. Amoebae were first observed histologically on week 16 (Table 7). The presence of *N. perurans* was confirmed by *in situ* hybridisation (Fig. 10A). Two further increases in gross pathology were observed in 2013 before a decline to minimal levels in January and February (weeks 36 – 44). Histopathology scores were recorded over the full range of the scoring method (Fig. 10 B-D) and at each sampling point some mild form of pathology was consistently observed. Amoebae were observed only on three occasions, on weeks 16 and 32 during the first and third AGD outbreaks and again on week 45 (Table 7).

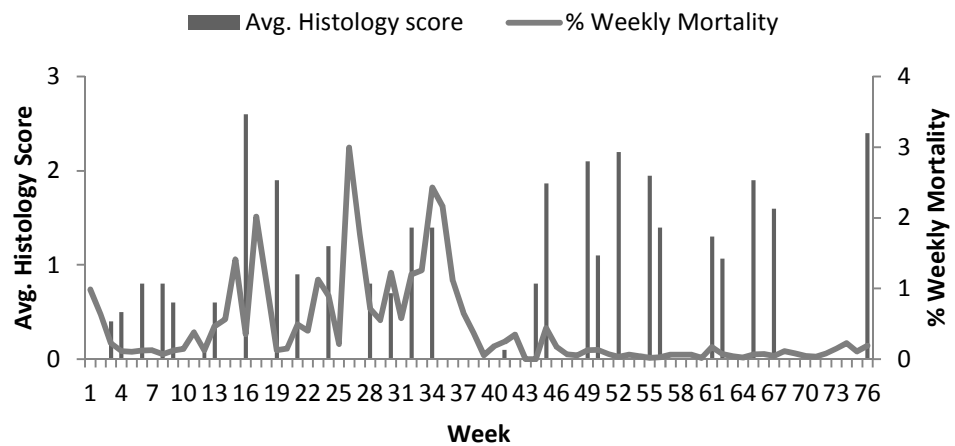
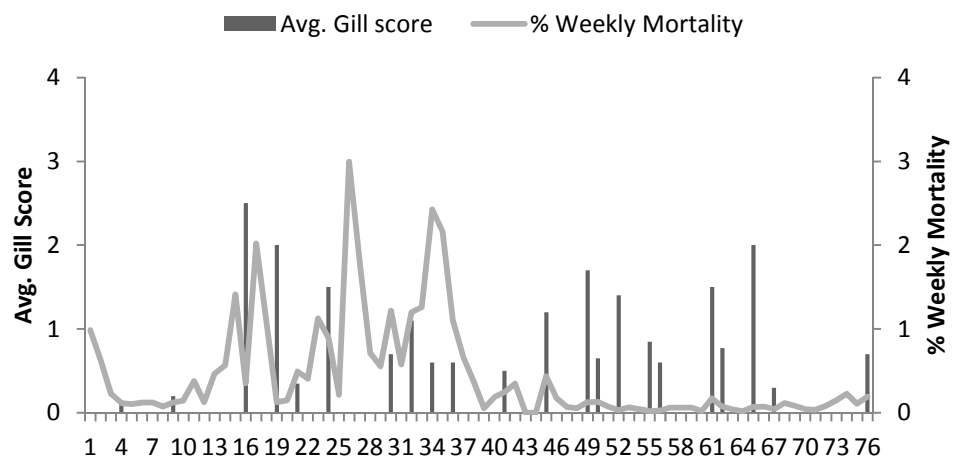
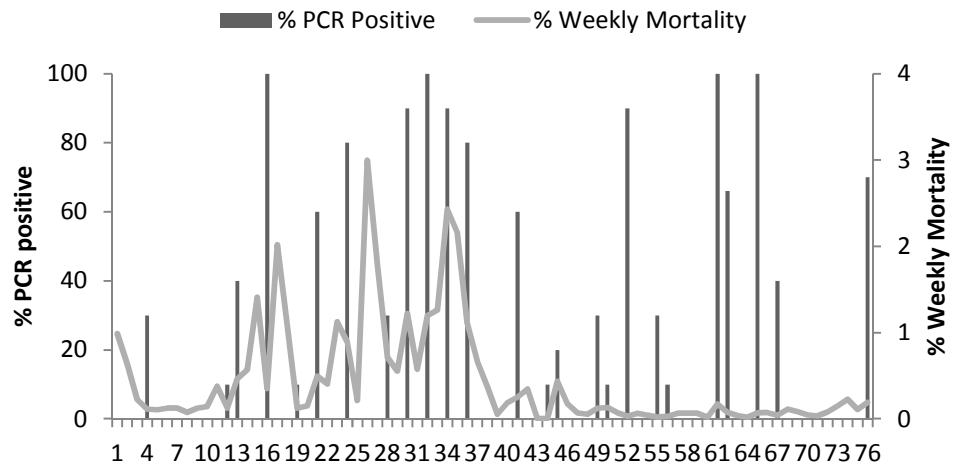


Figure 9. Weekly mortality rates versus (A) % PCR positive fish, (B) average gill scores, (C) average histology scores, throughout the marine production cycle during the longitudinal study.

Table 7. A summary of the Avg. gill score, Avg. histological score (with comments) and % PCR positive fish throughout the marine production cycle during the longitudinal study.

| Date | Week | Avg. gill score | Avg. histological score | Histological results | PCR results (% positive) |
|-------------|-------------|------------------------|--------------------------------|---|---------------------------------|
| 3/5/2013 | 4 | 0.1 | 0.5 | No amoeba, no gill pathology (or No evidence of AGD) | 30 |
| 17/5/2013 | 6 | 0 | 0.2 | No amoeba, no gill pathology | 0 |
| 31/5/2013 | 8 | 0 | 0.3 | No amoeba, no gill pathology | 0 |
| 7/6/2013 | 9 | 0.2 | 0.6 | No amoeba, no gill pathology | 0 |
| 28/6/2013 | 12 | 0 | 0.1 | No amoeba, no gill pathology | 10 |
| 05/7/2013 | 13 | 0 | 0.6 | No amoeba, no gill pathology | 40 |
| 26/07/2013 | 16 | 2.5 | 2.6 | Severe pathology consistent with AGD observed in all of the gills. Some amoeba observed | 100 |
| 16/08/2013 | 19 | 2 | 1.9 | No amoeba observed, Moderate gill pathology observed | 10 |
| 30/08/2013 | 21 | 0.35 | 0.9 | No amoeba observed, Low gill pathology observed | 60 |
| 20/9/2013 | 24 | 1.5 | 1.2 | No amoeba observed, Moderate gill pathology observed. Changes associated with AGD. | 80 |
| 18/10/2013 | 28 | 0 | 0.8 | No amoeba observed, Low to moderate gill pathology observed, significant telangiectasis | 30 |
| 01/11/2013 | 30 | 0.7 | 0.7 | No amoeba observed, Low gill pathology observed | 90 |

| Date | Week | Avg. gill score | Avg. histological score | Histological results | PCR results (% positive) |
|-------------|-------------|------------------------|--------------------------------|--|---------------------------------|
| 15/11/2013 | 32 | 1.1 | 1.4 | Low to severe levels of gill pathology, some amoeba observed | 100 |
| 29/11/2013 | 34 | 0.6 | 1.4 | Amoeba observed, low to moderate gill pathology observed | 90 |
| 13/12/2013 | 36 | 0.6 | N/A | N/A | 80 |
| 17/01/2014 | 41 | 0.5 | 0.1 | No amoeba observed. Low level gill pathology observed | 60 |
| 07/02/2014 | 44 | 0 | 0.8 | Low gill pathology observed | 10 |
| 14/07/2014 | 45 | 1.2 | 1.87 | Low to severe levels gill pathology, Amoeba observed. | 20 |
| 14/03/2014 | 49 | 1.7 | 2.1 | Low to severe levels gill pathology. Some old scarring evident | 30 |
| 21/03/2014 | 50 | 0.65 | 1.1 | Low to moderate gill pathology observed some very focal hyperplasia and fusion with old scarring. | 10 |
| 04/04/2014 | 52 | 1.4 | 2.2 | Moderate to severe gill pathology observed | 90 |
| 25/04/2014 | 55 | 0.85 | 1.95 | Moderate to severe gill pathology, some telangiectasia evident. | 30 |
| 02/05/2014 | 56 | 0.6 | 1.4 | Low to moderate gill pathology with some telangiectasia evident. Some bleeding also noted. | 10 |
| 06/06/2014 | 61 | 1.5 | 1.3 | Low to moderate gill pathology | 100 |
| 13/06/2014 | 62 | 0.77 | 1.07 | Mainly low level of pathology observed some with no significant findings. Some severe level of pathology with telangiectasia evident | 66 |

| Date | Week | Avg. gill score | Avg. histological score | Histological results | PCR results (% positive) |
|-------------|-------------|------------------------|--------------------------------|---|---------------------------------|
| 04/07/2014 | 65 | 2 | 1.9 | Moderate to severe gill pathology observed | 100 |
| 18/07/2014 | 67 | 0.3 | 1.6 | Low to Severe gill pathology observed, some telangiectasia evident | 40 |
| 19/09/2014 | 76 | 0.7 | 2.4 | Moderate to severe gill pathology observed with significant telangiectasia. | 70 |

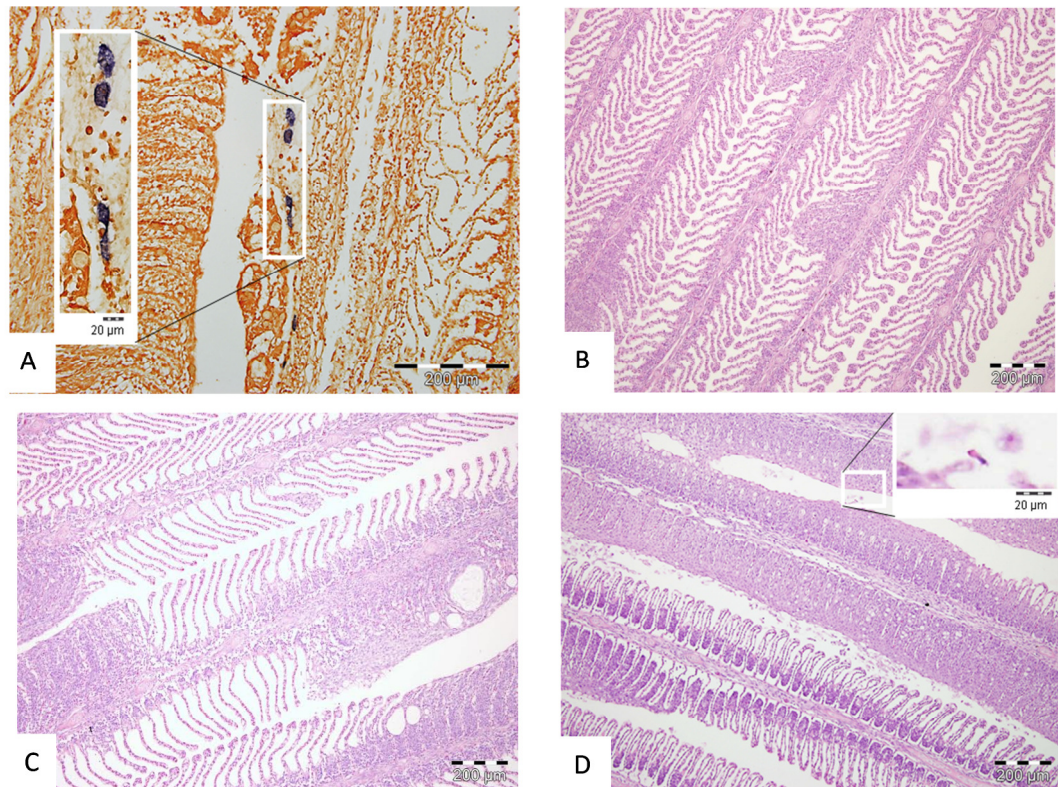


Figure 10. (A) *In situ*-hybridisation using species-specific oligonucleotide probes on Atlantic salmon gill sections examined from the study site. (Insert) Reactive dark cells indicate the presence of *N. perurans*; (B-D) Examples of the different levels of pathology observed in the gills of Atlantic salmon during this study; (B) a score of “1” was assigned to a section where < 10 % pathology was observed; (C) a histological score of “2” where there is between 10 – 50 % pathology observed; (D) gills with > 50 % pathology showing complete loss of structure due to hyperplasia and fusion and (insert) amoeba present (histological score of “3”).

2.5 Discussion

Over the last decade, gill pathologies have become an increasing problem for the Atlantic salmon aquaculture industry in Northern Europe (Rodger et al. 2011). In recent years, jellyfish, zooplankton, phytoplankton, bacteria, viruses and parasites have all been identified as causing fish kills and significant gill pathology in farmed salmonids (Mitchell & Rodger 2007, Doyle et al. 2008, Baxter et al. 2011), although, in terms of economic impact the most significant gill disease currently affecting the industry is AGD.

In 2011, AGD re-emerged as a significant disease of marine farmed Atlantic salmon in Ireland and has remained a major issue since then (Rodger 2014). The rapid detection of pathogens is essential for the implementation of an effective health management plan in aquaculture. This study aimed to develop a real-time PCR assay for the detection of *N. perurans* in Atlantic salmon gill samples and to

validate the assay as a surveillance tool for AGD through the marine grow-out phase of the production cycle. The assay reported in this study was designed to amplify a smaller (70 bp) segment of the *N. perurans* 18S rRNA gene than the one described by Fringuelli et al. (2012). The assay optimised for the detection of *N. perurans* was shown to have a high efficiency 98.44% and an R^2 value of 0.999, within the accepted levels of $100 \pm 10\%$ (Purcell et al. 2011) and was able to repeatedly detect as low as 2.68 copy numbers of *N. perurans* DNA μl^{-1} , which is at the theoretical limit of sensitivity for real-time PCR assays (Bustin et al. 2009).

Following optimisation, the assay was then utilised in a longitudinal study for the detection of *N. perurans* on a farm site in the south west of Ireland. Longitudinal studies have been used to investigate a range of diseases of importance in aquaculture such as heart and skeletal muscle inflammation (Kongtorp et al. 2006), pancreas disease (Graham et al. 2010) and also AGD (Clark & Nowak 1999). These studies provide important information on potential risk factors, impact of the disease and on the performance of diagnostic methods. This study covered the full marine production cycle from week four post-transfer (3rd May 2013) up until week 76 (19th September 2014). During this period, three peaks in mortality were recorded on the site, each one due to an outbreak of AGD, although the second mortality peak was also due to a large bloom of *Pelagia noctiluca*, known to cause significant pathology and mortality in farmed Atlantic salmon (Marcos-López et al. 2014). Mortality started to increase during week 13, at a time when the seawater temperature first rose above 15°C. There was an increase in the mean gill score and histological score on week 16, when pathology consistent with AGD as well as amoebae were observed on the gills. It is recognised that the histological gill score may have been influenced by the buffered formalin fixative used in this study and the use of an alternative fixative such as Davidson's may have resulted in higher retention of amoebae on the gills sampled (Cadoret et al. 2013), however, it was more practical to use buffered formalin which is also routinely used in fish histopathology. The sensitivity of the real-time assay was demonstrated by the fact that positive fish were already detected by week 12 (10% of fish tested were positive) and by week 16 all fish tested were positive for *N. perurans*.

Following the increase in mortality and diagnosis of AGD, the site undertook a freshwater treatment of every cage on week 18 to treat for the disease. Samples

collected four days post-treatment, during week 19, showed a reduction in the percentage of PCR-positive fish (10 %), which is in line with previous findings where a reduction in the number of amoebae was observed following freshwater bathing (Clark et al. 2003). Both the average gill and histological scores were reduced by week 21, as it can take up to four weeks post-treatment for gills to fully recover (Findlay et al. 2000). However, both gross and histological gill scoring can be misleading due to the presence of scarring from the previous infection, requiring adjustment in their interpretation. Due to some amoebae remaining and all cages not receiving treatments simultaneously, re-infection can occur as early as one week post-treatment and can increase in severity over the following weeks (Clark et al. 2003, Adams & Nowak 2004). This study confirmed a similar re-infection profile where an increase in the number of PCR positive fish by week 21, only three weeks following the first freshwater bath, was observed. Interestingly, it was six weeks post-treatment when an increase in the average gill score and histology score was observed and a second full site treatment was required on week 24. In total there were 6 recorded freshwater bath treatment events over the term of the study, the first 2 involving treatment of each cage on site while the 4 subsequent treatments were administered to a subset of specifically selected cages.

The PCR assay developed in this study was shown to have a beneficial role in monitoring the progress of the disease, in particular with detection of the amoeba three weeks prior to detection via gross pathology. The ability of this assay to detect amoebae a number of weeks prior to traditional diagnostics can potentially provide farm managers with valuable information to effectively plan treatments. Such information is important where infrastructure (well-boats) and the resources required (access to freshwater) for treatment are limited (Nowak 2012). As traditional detection methods require advanced stages of the disease in a greater proportion of the population, use of molecular based diagnostic tools could allow for earlier intervention strategies. Although the traditional screening methods (gill scores, wet-preparations and histology) are important tools for on-site monitoring of AGD, significant experience is required as amoebae can be difficult to differentiate from gill epithelial cells and observation of amoebae cells is not always possible, particularly when infection levels are low (Munday et al. 2001).

Interestingly, the level of weekly mortalities recorded during the outbreaks of AGD on the surveillance site were slightly lower than that observed in outbreaks of

AGD in Tasmania where levels of 2 – 4 % per week were recorded in fish weighing 1 - 2kg (Munday et al. 2001). In this study the weekly mortality peaked at just over 2 % in the first year of production when fish weighed less than 1 kg. Following a reduction in temperature in January and February 2014 and fish weights increasing above 1 kg, the percentage mortality returned to background levels for the remainder of the study.

This is the first study of AGD, conducted under field conditions over a full marine production cycle on a farm in Ireland. During this study, there were three separate outbreaks of AGD on this site. Each outbreak was preceded by a rise in the number of fish testing positive by PCR and subsequently by increased gill and histology scores. The development of an early detection method which is economical, sensitive and specific to diagnose AGD in the early stages of infection is an extremely valuable tool. As with other diagnostic methods further considerations are required and all on-site factors and observations must be taken into account when preparing a diagnosis (Munday et al. 2001). This was evidenced in the second year of production when PCR positive fish, gill scores and histology scores indicative of infection with *N. perurans* were recorded, although mortality levels remained low. While the immune response of Atlantic salmon to *N. perurans* is still poorly understood, there is some evidence to suggest that the fish which have survived an initial challenge of AGD develop some resistance or tolerance to the parasite (Vincent et al. 2006, Taylor et al. 2009, Valdenegro-Vega et al. 2015). This may also be influenced by differing genetic traits for the mechanisms which have been found to be involved in the resistance to the first and subsequent infections (Kube et al. 2012).

In conclusion, the assay developed in this study demonstrated potential as a tool to complement existing techniques for monitoring AGD. Future studies, utilising non-lethal gill swabs will further enhance monitoring capabilities for AGD by the aquaculture industry.

2.6 Author Contributions

Conceived and designed the experiments: Jamie Downes, Kathy Henshilwood, Eugene MacCarthy, and Neil Ruane. Performed the experiments and sample analysis: Jamie Downes, Abdon Ryan, and Evelyn Collins. Writing and editing

manuscript: Jamie Downes, Kathy Henshilwood, Eugene MacCarthy, Ian O'Connor, Hamish Rodger and Neil Ruane.

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Chapter 3

Evaluation of non-destructive molecular diagnostics for the detection of
Neoparamoeba perurans

This chapter is a verbatim reproduction from the following published paper:

Downes JK, Rigby ML, Taylor RS, Maynard BT, MacCarthy E, O'Connor I, Marcos-Lopez M, Rodger HD, Collins E, Ruane NM and Cook MT (2017) Evaluation of non-destructive molecular diagnostics for the detection of *Neoparamoeba perurans*. *Frontiers of Marine Science* 4:61 doi.org/10.3389/fmars.2017.00061

3.1 Abstract

Amoebic gill disease (AGD) caused by *Neoparamoeba perurans*, has emerged in Europe as a significant problem for the Atlantic salmon farming industry. Gross gill score is the most widely used and practical method for determining AGD severity on farms and informing management decisions on disease mitigation strategies. As molecular diagnosis of AGD remains a high priority for much of the international salmon farming industry, there is a need to evaluate the suitability of currently available molecular assays in conjunction with the most appropriate non-destructive sampling methodology. The aims of this study were to assess a non-destructive sampling methodology (gill swabs) and to compare a range of currently available real-time PCR assays for the detection of *N. perurans*. Furthermore a comparison of the non-destructive molecular diagnostics with traditional screening methods of gill scoring and histopathology was also undertaken. The study found that all molecular protocols assessed performed well in cases of clinical AGD with high gill scores. A TaqMan® based assay (protocol 1) was the optimal assay based on a range of parameters including % positive samples from a field trial performed on fish with gill scores ranging from 0 to 5. A higher proportion of gill swab samples tested positive by all protocols than gill filament biopsies and there was a strong correlation between gill swabs tested by protocol 1 and gross gill score and histology scores. Screening for *N. perurans* using protocol 1 in conjunction with non-destructive gill swab samples was shown to give the best results.

Keywords: Atlantic salmon, amoebic gill disease, *Neoparamoeba perurans*, molecular diagnostics, method validation

3.2 Introduction

Amoebic gill disease (AGD), caused by *Neoparamoeba perurans*, is a major health challenge for the global Atlantic salmon (*Salmo salar*) farming industry (Rodger, 2014; Oldham et al., 2016). AGD has affected the marine Atlantic salmon industry in Tasmania since the 1980's and has since been described in farmed salmon in Ireland (Rodger and McArdle, 1996), Norway (Steinum et al., 2008), Chile (Bustos et al., 2011) as well as France, Scotland and the Faroe Islands (Rodger, 2014, Oldham *et al.*, 2016). In addition to Atlantic salmon, AGD has also been described in a number of other marine fish species (Oldham et al., 2016) including cleaner fish species used as a biological control of sea lice in Atlantic salmon farms (Haugland et al., 2017).

If left untreated, AGD can cause significant mortality, up to 10% of livestock per week (Munday et al., 2001). The economic cost of a challenging issue is often the catalyst with regards to prioritising research and the management of resources (Costello, 2009). Current AGD management practices are resource demanding and labor intensive, involving numerous freshwater bath treatments throughout a production cycle. Freshwater bathing has been the standard method of treating the disease in Tasmania but is limited by access to freshwater (Nowak et al., 2014). In cooler production areas, hydrogen peroxide is an effective treatment, but the treatment is recognised as having a narrow safety margin at higher temperatures (Adams et al., 2012) or where fish are compromised by advanced AGD (McCarthy et al., 2015). Some estimates have put the cost of AGD-related mortality between \$12.55 million in Norway and \$81 million in Scotland (Shinn et al., 2015).

The case definition for AGD is through histopathology, where amoebae are observed with associated pathology (Clark and Nowak, 1999; Rodger, 2014). By far the most widely used and practical method for ascertaining AGD severity and hence triggers for intervention (freshwater bathing, hydrogen peroxide treatments) is the gross gill score across all 16 hemibranchs, as described by Taylor et al. (2009), which may be coupled with histopathology and fresh microscopy to confirm the presence of lesion-associated amoebae. The identification of *N. perurans* as the causal agent of AGD (Young et al., 2007) has allowed the development of specific DNA based molecular diagnostic assays for the detection of the amoeba. Currently there are two conventional polymerase chain-reaction (PCR) assays published for the detection of *N. perurans*, (Young et al., 2008;

Rozas et al., 2011), while three real-time PCR assays were developed based on SYBR® Green (Bridle et al., 2010) and TaqMan® chemistries (Fringuelli et al., 2012; Downes et al., 2015).

A standardised molecular diagnostic method has the potential to fulfil a role as an early warning and monitoring tool which would greatly complement traditional diagnostic methods, particularly in the early stages of infection when gross clinical signs may be absent and in other fish species for which the gill scoring method is less applicable. The aims of this study were to compare two non-destructive methods of sampling for *N. perurans* to confirm AGD, gill swabs and gill filament biopsy samples taken from the same animal during a naturally occurring infection in a field trial. A range of currently available molecular assays for the detection of *N. perurans* were compared with regards to sensitivity, specificity and practicality, utilising the samples taken from the field trial. Furthermore, the preferred molecular assay used to test gill swab samples was then compared with the traditional screening methods of gross gill scoring and histopathology, on samples taken during an experimental infection trial.

3.3 Materials and Methods

3.3.1 Field Trial

The field trial consisted of commercial all-female diploid Atlantic salmon, which were put to sea as smolts in south east Tasmania, Australia, on the 8th of July 2014. The samples were collected on the 13th of March 2015 when the fish were an average weight of 1.5 kg. All fish had previously been subjected to 5 freshwater bath treatments, the last of which was on 25th of February 2015. At the time of sampling, biomass in the cage was 96,407 kg (5.7 kg m⁻³). Five fish were selectively sampled from each gill score (scores 0-5 assessed across all gill surfaces, Taylor *et al.*, 2009) (n=30) with individual scores being recorded for each fish. The second gill arch on either side of each fish was used for tissue sampling, a gill filament biopsy was conducted on the right side targeting 25 ± 2 mg per sample while the front and back of the left side second arch was swabbed with isohelix swabs (Cell Project Ltd.). Both the filament and swab samples were stored in 2 mL screw-cap micro-centrifuge tubes in 100% AR ethanol for transport and storage.

3.3.2 Sample preparation and DNA extraction

Swab samples were placed into a tissue lyser (Qiagen) for 10 min at a frequency setting of 20.0 Hz before vortexing and pulse centrifuging of each individual tube. The swabs were removed using a sterile forceps taking care not to cross-contaminate samples. Both swab and filament samples were then spun down at 21,130 g for 10 min in order to form a visible pellet (for swab samples) and to facilitate ethanol removal. Extraction was then completed using the DNA easy mini kit (Qiagen) according to manufacturer's instructions.

3.3.3 Real-Time PCR (qPCR) evaluation and protocols

The published methodologies available at the time of evaluation are listed in Table 8. Qualitative analysis of each assay was assessed in relation to the percentage of positive results for the gill swabs and filament samples in the field trial. The sensitivity, specificity, linearity and correlation to gill score of each assay were also analyzed. In order to investigate the sensitivity of the assays, a plasmid was created and its concentration determined as previously described (Downes et al., 2015). A dilution series was generated and analyzed by the three real-time assays (protocols 1, 2 and 4) to assess the lowest copy numbers detectable. Only dilutions that produced Ct values in all triplicates were included in the analysis. DNA extracted from *in vitro* cultures of *N. perurans* obtained from three countries (Norway, Ireland and Australia) were used to assess the specificity of each of the assays. Additionally, the assays were appraised with respect to cost (in AUS\$) per sample (cost of reagents for each assay for a single sample run in triplicate) and time requirements (runtime for each method in relation to through-put). All results were reviewed and each assay was then ranked on a scale of 1 to 5, with 1 being the most optimal score. These scores were combined in order to compare the assays.

For each protocol, primers were obtained from Sigma (for work completed in Ireland) or GeneWorks (for work completed in Australia). TaqMan® probes and master mix for protocols 1 and 2 were purchased from Life Technologies.

3.3.3.1 Protocol 1

Protocol 1 is a TaqMan[®] qPCR targeting the 18s rRNA gene sequence of *N. perurans* generating an amplicon of 70 bp (Downes et al., 2015).

3.3.3.2 Protocol 2

Protocol 2 is a TaqMan[®] qPCR targeting the 18s rRNA gene sequence of *N. perurans* generating an amplicon of 139bp (Fringuelli et al., 2012).

3.3.3.3 Protocol 3

Protocol 3 was a commercial kit developed by Primerdesign Ltd for the detection of *N. perurans*. Each reaction mixture and thermal profile was completed following manufacturer's instructions.

3.3.3.4 Protocol 4

Protocol 4 was a SYBR[®] Green protocol targeting the 18s rRNA gene sequence of *N. perurans* generating an amplicon of 146 bp (Bridle et al., 2010). Each qPCR reaction contained 0.4 μ M of each primer, 2x SensiFAST SYBR[®] Lo-ROX Master Mix (Bioline) and nuclease-free water. Following optimisation of this protocol (unpublished) it was determined that addition of neat DNA was not appropriate, resulting in Ct values < 10, therefore samples were diluted to ~ 5 ng μ l⁻¹ and 2 μ l (10 ng) of DNA was added to each reaction.

The reaction was incubated at 95 °C for 10 min followed by 45 cycles of 95 °C for 10 sec, 60 °C for 15 sec and 72 °C for 34 sec. Following the 45 cycles a melt curve analysis was performed to determine the specificity of the reaction. A 5-point standard curve based on a known quantity of cultured amoebae (100, 50, 25, 10, 5 and 1 cell and also diluted down to 5 ng μ l⁻¹) was included in each run. To validate the results the melting temperature of the qPCR products were compared with the T_m of the culture (T_m ~ 77 °C). A sample was considered positive if the T_m was between 75-77.5 °C.

3.3.3.5 Protocol 5

Protocol 5 was a modified nested PCR based on the first round amplification described by (Young et al. 2008) which amplifies a 636 bp region of the *N. perurans* 18S rRNA gene followed by a 1:5 dilution of the PCR product in nuclease-free water and analyzing further using protocol 4. The nested PCR consisted of 0.365 μM of each primer, 2x GoTaq[®] Colorless Master Mix (Promega) and nuclease-free water. This initial amplification was completed for all samples, the full range of the known cell standard curve (100, 50, 25, 10, 5 and 1 cell) and also a Nested No Template Control (Nested NTC). As in Protocol 4, samples and the standard curve were diluted to 5 $\text{ng } \mu\text{l}^{-1}$ and 2 μL (10 ng) of DNA added.

For each protocol, all samples were run in triplicate on an Applied Biosystems AB7500 Real-Time instrument and associated software. Each run included a positive control, a negative control and a non-process control. An external process control (salmonid elongation factor-1 α ; Bruno et al., 2007) was used for each sample.

Table 8. A list of the real-time PCR protocols evaluated in this study in addition to the salmon elongation factor assay used as an external process control. Protocol 3 is commercially available as a kit and does not include information on the primer/probe sequences.

| Protocol | Gene | Target | Primer/Probe | Name | Sequence (5' - 3') | Product Size (bp) | Reference |
|--------------------------|----------|--------------------|--------------|---------|----------------------------------|-------------------|------------------------|
| 1 | 18S rRNA | <i>N. perurans</i> | For | NP1 | AAAAGACCATGCGATTTCGTAAGT | 70 | Downes et al. 2015 |
| | | | Rev | NP2 | CATTCTTTTCGGAGAGTGGAAATT | | |
| | | | Probe | NPP | 6-FAM- ATCATGATTCACCATATGTT-MGB | | |
| 2 | 18S rRNA | <i>N. perurans</i> | For | Peru.F | GTTCTTTTCGGGAGCTGGGAG | 139 | Fringuelli et al. 2012 |
| | | | Rev | Peru.R | GAACTATCGCCGGCACAAG | | |
| | | | Probe | Peru.P | 6-FAM-CAATGCCATTCTTTTCGGA-MGB | | |
| 4 | 18S rRNA | <i>N. perurans</i> | For | QNperF3 | GTTTACATATTATGACCCACT | 146 | Bridle et al. 2010 |
| | | | Rev | QNperR3 | TAAACCCAATAGGTCTGC | | |
| 5 | 18S rRNA | <i>N. perurans</i> | For | Npr.F | ATCTTGACTGGTTCTTTTCGGGA | 636 | Young et al. 2008 |
| | | | Rev | Nper.R | ATAGGTCTGCTTATCACTYATTCT | | |
| External Process Control | ELF | Salmonid | For | S-ELF.F | GGCCAGATCTCCCAGGGCTAT | 66 | Bruno et al. 2007 |
| | | | Rev | S-ELF.R | TGAACTTGCAGGCGATGTGA | | |
| | | | Probe | S-ELF.P | 6-FAM-CCTGTGCTGGATTGCCATACTG-MGB | | |

3.3.4 Amoebae culture

In order to conduct an infection trial for the comparison of the preferred assay with traditional screening methods, *N. perurans* was isolated from farmed Atlantic salmon affected by AGD in the west of Ireland using a method described in Downes et al. (2015), adapted from Morrison et al. (2004). The amoeba culture was established and maintained according to Crosbie et al. (2012). To confirm the presence of *N. perurans* in the culture, a sub-sample of the culture was tested by conventional PCR (Young et al., 2008). Amoebae were harvested by physical removal from the agar using a bacteriological spreading bar, followed by several seawater washes. The amoeba seawater solution was then collected in a sterile flask. Several counts of the amoeba seawater solution were undertaken using a 1 ml Sedgewick Rafter Counting Chamber (SPI Supplies).

3.3.5 Infection Trial

The infection trial was carried out at the Daithi O’Muruchu Marine Research Station, Bantry, Co. Cork, Ireland using four 400 L flow-through tanks at full salinity which were each stocked with 50 Atlantic salmon smolts weighing approximately 70 g. Following an acclimation period of two weeks, two of the tanks were challenged with cultured *N. perurans* and two other tanks were used as negative controls. For the infected tanks, the water level was lowered and inoculated with amoeba at 1,000 cells L⁻¹ for 4 hrs. Throughout the trial the fish were fed 1 % of body weight per day, water quality was monitored daily and the temperature was constant at 11-12 °C throughout the trial. This work was authorised by the Health Products Regulatory Authority (HPRA) in Ireland under project authorisation number AE19114/P001, following the Animals Scientific Procedures Act 1986 (Directive 2010/63/EU transposed into Irish law by S.I. No 543 of 2012).

Sampling commenced 24 h post-infection with further samples taken at 2, 3, 8, 15 and 21 d post-infection. At each sampling point, three fish were sampled from each tank. Gross gill scoring of individual fish (n = 3) was conducted onsite using the gill scoring system described by Taylor et al. (2009). The second gill arch on the right-hand side was swabbed (Isohelix) and processed as described above for the field trial. Gill swab samples were tested by real-time PCR (Protocol 1). The second gill arch on the left-hand side was excised from

each fish and immediately fixed in 10% neutral buffered formalin for histological processing. Sections (5 µm) from paraffin embedded tissue were stained with haematoxylin and eosin and examined microscopically on an Olympus BX51 microscope. In order to determine if there was a correlation between gross pathology and histopathology, a scoring system based on that described by Mitchell et al. (2012) was applied to determine the progression and severity of gill lesions in fish where AGD developed. A score of 0 = normal gill; 1= low pathology <10% of gill tissue affected; 2 = moderate pathology <50% of gill tissue affected and 3 = severe pathology > than 50% of gill tissue affected. Only histological sections where pathology was observed in the presence of amoeba were recorded as AGD infected (Clark & Nowak 1999; Rodger 2014).

3.3.6 Statistical analysis

Regression analysis was carried out in order to determine the lowest detectable copy number of each of the assays (Microsoft Excel). Spearman's correlation analysis was undertaken to assess the relationship between gross gill pathology scores, histopathology scores and PCR analysis (Minitab 17). Kappa statistics were conducted in order to determine the level of agreement between PCR analysis, gill scoring and histopathology.

3.4 Results

3.4.1 Field Trial

PCR results of each of the five protocols for both gill swabs and filaments are shown in Fig. 11. Overall, more positive results were detected by each protocol for gill swab samples compared with gill filament biopsies. Protocol 1 gave 100 % positive gill swabs, down to 55 % with protocol 4. Protocol 1 gave 79 % positive results with gill filament biopsy, compared with 14 % for protocols 3 and 4.

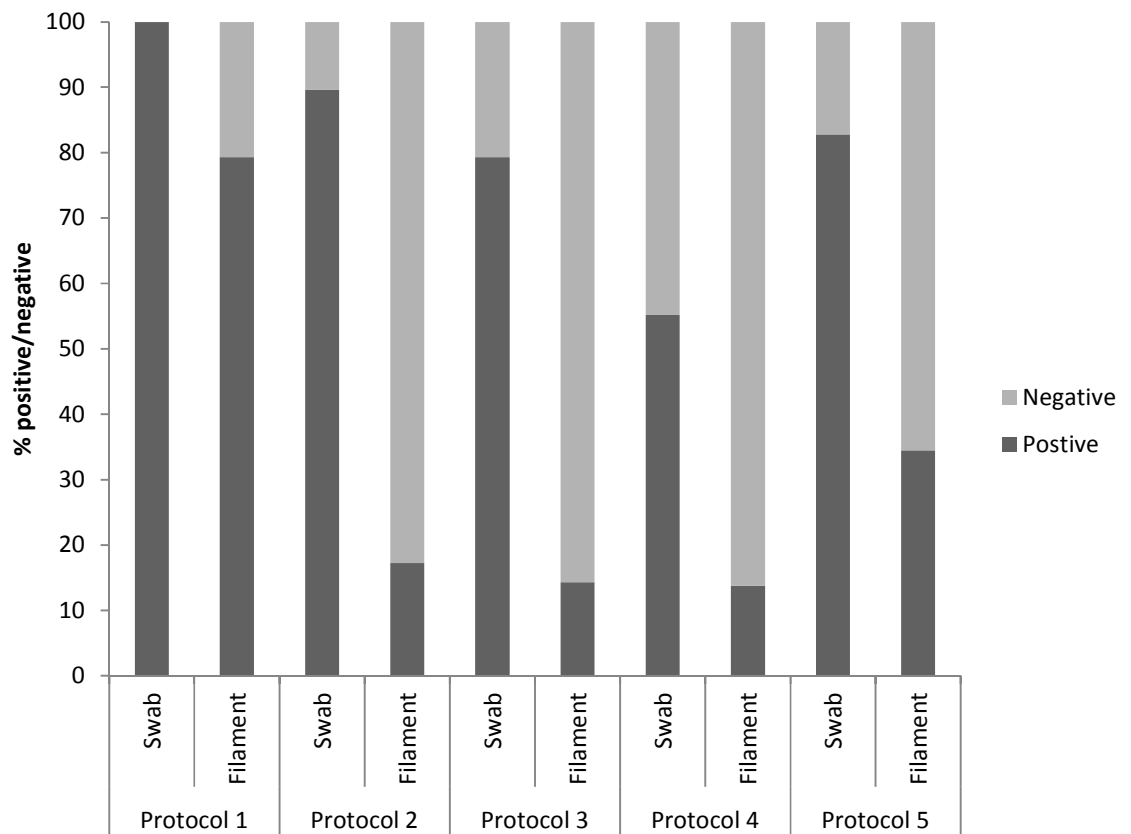


Figure 11. The percentage of gill swab and gill filament biopsy samples which were positive/negative for *Neoparamoeba perurans* during a natural outbreak of amoebic gill diseases in Atlantic salmon during a field trial, assessed by five qPCR protocols.

The percentage positive results for each protocol, for both gill swab and gill filament biopsy, across each gross gill score (0 – 5) are shown in Table 9. A higher number of positive results were found when using gill swabs rather than filament biopsies. There is a general increase of positive swab samples with increasing gross gill score, all protocols had 100 % positive results at gill score 5 whereas only protocols 1 and 3 gave 100 % positive results at gill scores 0 – 3. In relation to the percentage of positive filament biopsy samples, only protocol 1 found 100 % of samples positive at gill scores of 2, 4 and 5.

Table 9. The percentage of positive results for each protocol for both gill swab and gill filament biopsy samples in relation to gross gill scores in Atlantic salmon during a field trial undergoing natural outbreaks of amoebic gill disease.

| Gross gill score | Swab | | | | | Filament | | | | |
|---------------------------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|
| | Protocol 1 | Protocol 2 | Protocol 3 | Protocol 4 | Protocol 5 | Protocol 1 | Protocol 2 | Protocol 3 | Protocol 4 | Protocol 5 |
| 0 | 100 | 80 | 100 | 20 | 80 | 80 | 0 | 0 | 0 | 0 |
| 1 | 100 | 80 | 100 | 60 | 60 | 40 | 0 | 20 | 0 | 0 |
| 2 | 100 | 80 | 100 | 20 | 60 | 100 | 20 | 20 | 0 | 20 |
| 3 | 100 | 100 | 40 | 60 | 100 | 60 | 0 | 20 | 0 | 40 |
| 4 | 100 | 100 | 80 | 80 | 100 | 100 | 60 | 20 | 60 | 80 |
| 5 | 100 | 100 | 100 | 100 | 100 | 100 | 40 | 0 | 40 | 80 |

3.4.2 Linearity, efficiency, sensitivity and specificity

The known numbers of cells examined was 25, 10 and 1 amoebae cells and were analyzed by protocols 1, 2, 4 and 5 only. Each assay performed well with multiple cells, only protocols 1 and 2 were found to reliably detect *N. perurans* down to a single cell (Table 10). Both protocol 1 and 2 provided very similar results for the known quantity of cells and were shown to perform best when analyzing a single cell with average Ct values of 35.10 and 35.51 respectively. These two assays also had similar linearity (R^2) values of 0.9679 (Protocol 1) and 0.9605 (Protocol 2), while protocol 5 had the lowest R^2 value of 0.8171 (Table 10). Three of the assays were analyzed in relation to the lowest detectable copy numbers, the limit of detection (LOD) determined for protocol 1 was 2.64 copies, protocol 2 was 14.7 and protocol 4 was 115 (Table 11). DNA extracted from cultures of *N. perurans* provided from three countries (Ireland, Norway and Australia) were analyzed to determine specificity and each protocol was found to perform comparatively (all results were positive). The amplification efficiency for each of the assays were found to be comparable and within the expected range of Ex = 90-110%.

Table 10. Ct values for known numbers of *N. perurans* cells and linearity assessed for protocols 1, 2, 4 and 5 (values are mean \pm SD).

| | R^2 value | Known No. Of Cells | | |
|-------------------|-------------|--------------------|------------------|-------------------|
| | | 25 | 10 | 1 |
| Protocol 1 | 0.9679 | 31.26 \pm 0.28 | 32.37 \pm 0.15 | 35.10 \pm 0.75 |
| Protocol 2 | 0.9605 | 31.29 \pm 0.26 | 32.35 \pm 0.27 | 35.51 \pm 0.51 |
| Protocol 4 | 0.8885 | 30.89 \pm 0.1 | 31.24 \pm 0.49 | 36.43 \pm 0.17* |
| Protocol 5 | 0.8171 | 26.05 \pm 0.17 | 27.27 \pm 0.25 | 39.39 \pm 1.05* |

*Ct values above the stated limit of detection for these assays.

3.4.3 Swab/Gill score correlation

There was a significant ($P < 0.01$) negative correlation for protocols 1, 2, 4 and 5 when analyzing the Ct values from the swab samples with the individual gross gill scores of the fish in the field trial (Table 11). The correlation between gross gill scores and gill filament

samples was found to be significant for protocols 1, 2 and 5 ($P < 0.01$), however, the correlation coefficient for each of the assays was noticeably lower for the gill filament samples when compared with gill swab samples (Table 11).

3.4.4 Cost and Time analysis

In relation to cost per sample, protocol 4 was the cheapest assay at \$2.76 AUD and protocol 3 the most expensive at \$24.38 AUD (Table 11). With respect to the time required based on a full 96-well plate, protocols 1, 2 and 3 take the same amount of time for analysis at 2h 55min, while protocol 5 took 6h 20min.

3.4.5 Ranking

All of the results recorded for each of the parameters examined were compared and ranked from 1 to 5 (1 being the optimal) (Table 11). It was found that protocol 1 performed better for several of the parameters such as PCR efficiency for known cell numbers, % of positive samples for swabs and filaments, % difference between the positive results for swabs and filaments, in addition to having the greatest correlation between swabs and gross gill score. Protocol 5 was ranked 1 for correlation in relation to the filaments and the gill score. Protocol 4 was ranked 1 in relation to cost.

Table 11. Ranking of the real-time PCR protocols on a range of parameters assessed by analyzing samples of naturally infected Atlantic salmon in a field trial.

| Protocol | Sensitivity | | | Correlation to gill score | | Additional factors | | | Overall Ranked by Median |
|----------|--------------------|---------------------------------------|--|-------------------------------------|----------------------------|--------------------------------|-------------------------------|-----------------|--------------------------------|
| | % +ve Filaments | % difference Swabs vs Filaments | Linearity of assay (R ²) | DNA copy no. (cells μ^{-1}) | SWABS (R ²) | FILAMENTS (R ²) | Cost per Sample (AUD\$) | Time (h:min) | |
| 1 | 79 | 21 | 0.9679* | 2.64 | -0.689* | -0.608* | \$6.50 | 2:55 | 1.0 |
| 2 | 17 | 82 | 0.9605* | 14.3 | -0.666* | -0.48* | \$7.36 | 2:55 | 2.5 |
| 3 | 14 | 82 | N/A | N/A | -0.109 | -0.069 | \$24.38 | 2:55 | 4.0 |
| 4 | 14 | 75 | 0.8885* | 115 | -0.656* | -0.445* | \$2.76 | 4:45 | 3.5 |
| 5 | 34 | 59 | 0.8171* | N/A | -0.606* | -0.618* | \$3.18 | 6:20 | 3.0 |

* P<0.05

3.4.6 Infection trial: Comparison of gill swab, histology and gross gill score

Results from the infection trial are shown in Fig. 12. Using protocol 1, the first PCR positive samples were detected 2 d post-infection (16 %) and by 15 d post-infection 100 % of the samples were positive. Both gill score and histology scores first increased above score 0 on 8 d post-infection and continued to increase throughout the remainder of the trial. Gross pathology, as characterised by white mucoid spots and plaques on the gill surface, was first recorded 8 d post-infection. Amoebae were first observed during histological examination 15 d post infection. There was a significant correlation ($P>0.01$) between each of the methods analyzed (Table 12). There was a significantly negative correlation between the PCR results and both the gill score (-0.938) and, the histology score (-0.836). Conversely, analysis between the gill score and histology score expressed a significant strong positive correlation (0.849). There was excellent concordance between the PCR and gill score ($K=0.80$) and between gill score and histology score ($K=0.80$). *N. perurans* DNA was detected in all samples where AGD was microscopically diagnosed, while overall agreement between the PCR and histology score was good ($K=0.69$).

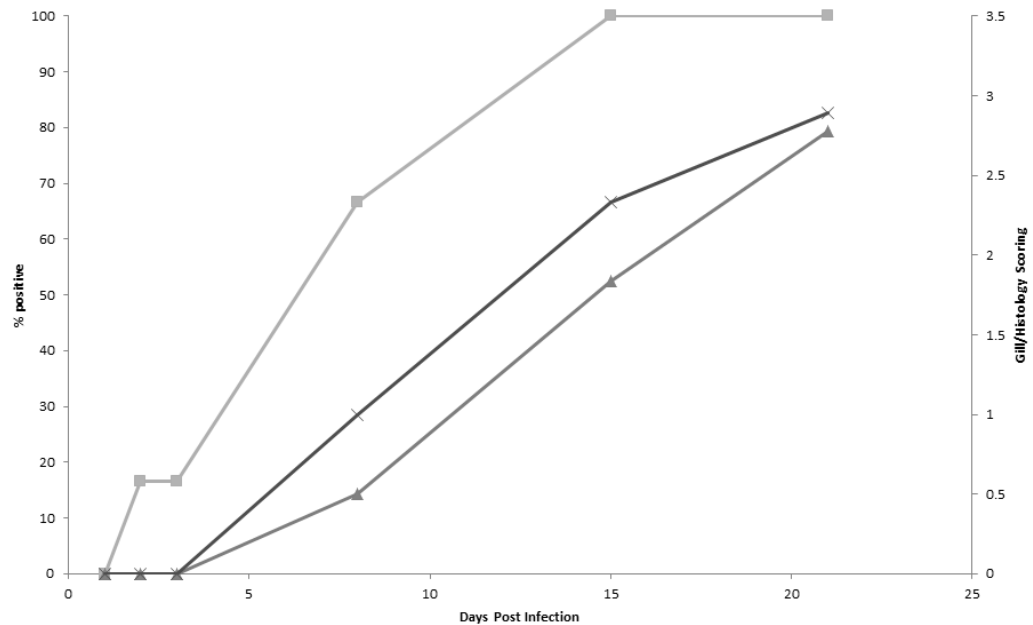


Figure 12. A comparison of qPCR positive results (protocol 1) (■), gross gill score (▲) and histology score (◆) in Atlantic salmon experimentally infected with *Neoparamoeba perurans*.

Table 12. Spearman's correlation between gill swab qPCR results, histology score and gross gill scores in Atlantic salmon experimentally infected with *Neoparamoeba perurans*.

| | Gill score | qPCR |
|------------------|------------|--------|
| qPCR | -0.938 | |
| Histology scores | 0.849 | -0.836 |

3.5 Discussion

The identification of *N. perurans* as the causative agent of AGD (Young et al., 2007; Crosbie et al., 2012) has allowed the development of a range of molecular based diagnostic assays for the detection of the amoeba. While it is acknowledged that alternative molecular diagnostic assays are available (Haugland et al., 2017; Hellebø et al., 2017) they have not been published in the literature and therefore could not be included in this study. Therefore this study has compared a range of published molecular assays available at the time and has shown that gill swab samples are more sensitive than gill filament biopsies, resulting in higher number of positive results from known infected fish. Molecular based diagnostic methods were also shown to correlate well with the more traditional diagnostic methods of gill and histology scoring.

Regular gross gill scoring provides fish farmers with immediate information on AGD prevalence and intensity to support husbandry and treatment decisions. This method is particularly suitable on Atlantic salmon farms that are constantly affected by the disease, allowing operators to become familiar with the gross presentation of lesions. However, the gross gill score can be difficult to interpret when non-AGD pathologies, such as proliferative gill disease or gill necrosis are present (Steinum et al., 2010; Mitchell and Rodger, 2011). It is also reported that the gross gill scoring method is less applicable for other fish species affected by AGD, such as lumpfish (*Cyclopterus lumpus*) which are used as cleaner fish in Atlantic salmon cages (Haugland et al., 2017).

It is clear from the results of this study that gill swabs displayed improved the sensitivity in comparison to gill filament biopsies. Results collected from each of the PCR protocols demonstrated an increase in the number of positive samples detected when samples were taken using the swabs. In addition to the increase in positive

detections, there was also a higher correlation between gill swab and gross gill scores across the majority of the assays. The difference observed between the two sampling methods (swabs vs filament) is likely due to the greater gill surface area sampled by the swab and there may be a greater abundance of amoeba in the mucous collected on the swab than between the distal filaments. However, one parameter that is likely to have had an effect on the difference of sensitivity between the two sampling methods is that of the matrix effect, where the presence of inhibitors affect the sensitivity of the assays (Schrader et al., 2012). There was an observed reduction in the amount of salmon DNA between the gill filament biopsy and the gill swabs when tested using the salmon ELF assay (data not shown). It must be noted that non-detection with some of the assays may be due to the quantity of amoebae DNA below the detection threshold of the assay rather than a technical failure of the assay (Collins et al., 2016)

The qualitative analysis of each protocol demonstrated differences between the positive/negative results produced. As the fish sampled during the field trial were taken from a naturally infected population, which was in its sixth round of AGD infection, it enabled the study to sample a broad range of AGD gross gill scores. The TaqMan® assays (protocols 1 & 2) produced relatively similar results with respect to the swabs. Conversely, there was a stark difference between the results produced for the filament samples using these two protocols. A shorter amplification fragment and with primers designed closer to the probe in protocol 1 appears to have increased the sensitivity of the assay thus ensuring more positive results when testing the filament samples. The effect of a smaller amplicon size has previously been found to be advantageous for TaqMan® assays in the presence of inhibiting compounds (Opel et al., 2010).

When using protocol 4, the melt curve analysis produced multiple peaks, which suggests a composite of more than one product with melting temperatures considerably different to that of the standard/positive control. Samples that produced Ct values but also a product with a different melting temperature were deemed to be negative. When analyzing the samples with protocol 5 (which was a modified protocol 4), no additional peaks were found for the swab samples and only 20 % of the filament biopsy samples produced additional peaks in the melt curve analysis suggesting that the nested PCR (protocol 5) appears to increase the specificity of the SYBR® assay (protocol 4). Multiple peaks found in melt curves of SYBR® Green

analysis is generally indicative of non-specific amplification. As SYBR® Green is a dye that binds to all double-stranded DNA molecules, the specificity of an assay based on this chemistry is due only to the choice of primers (Martenot et al., 2010). Consequently, mispriming and the formation of primer dimers can produce false positive results in addition to overestimates of DNA quantities (Bustin, 2000). In other comparisons of SYBR® Green and TaqMan® chemistries where there are low copy numbers of the gene, there is a greater accumulation of primer dimers and non-specific double stranded DNA by the SYBR® Green Chemistry (Hein et al., 2001; Martenot et al., 2010). Additionally, TaqMan® chemistry is generally thought to offer a number of advantages over SYBR® Green, in particular, the incorporation of minor-groove-binders (MGB) which allow for the raising of melting temperatures of the probes (enabling the use of shorter probes) and integration of the internal hydrolysis probe providing greater specificity in comparison to the intercalating dye assays, which have reduced specificity because any amplification product incorporates the dye (Gunson et al., 2006; Purcell et al., 2011).

Each assay was designed to amplify specific regions of the 18S rRNA gene, which is generally chosen due to its high copy number, thus potentially increasing sensitivity. The 18S rRNA gene is an established marker for microbial identification and has been utilised in numerous studies due to the availability of a large database of species specific sequences (Bridle et al., 2010). Another reason for this choice is that multiple copies of this gene are encoded within the eukaryotic genome (Long and Dawid, 1980; Young et al., 2008). Sensitivity in relation to the lowest detectable DNA copy numbers was assessed for protocols 1, 2 & 4. Two of the protocols were not assessed for DNA copy numbers due to unavailability of information on the primer sequences for protocol 3 and for protocol 5, which was based on the Young et al. (2008) primers, used to produce the plasmid DNA. From the analysis of the three protocols assessed it was found that protocol 1 was able to detect the lowest concentration of copies of DNA at 2.64 copies μl^{-1} , which approaches the theoretical limit of detection (Purcell et al., 2011). Analysis of protocol 2 gave a concentration of 14.3 copies μl^{-1} which is similar to levels reported by Fringuelli et al. (2012). Protocol 4 has previously been described as having an LOD of 1.418 copies μl^{-1} , however this was not achievable during this study and was found to be able to detect

115 copies μl^{-1} . Further dilutions produced multiple peaks and incorrect melting temperatures, making melt curve analysis difficult.

Correlations between the molecular results and the gill scores were consistent for each of the protocols used during the field trial for both gill swabs and filament biopsies. Previously Bridle et al., (2010) reported excellent agreement between AGD gross gill scores (Powell et al., 2001) and molecular results (gill scores 1 through to 4, no gill score 0), though the gill area sampled, fish size and season were not specified. The results of the current field study showed lower agreement, which may reflect the wider gill score range used in this study (from 0 to 5). The samples were taken in early Autumn (Australia), which is typically associated with a slowing of AGD pathology and the onset of non-specific gill necrosis prior to Winter. A higher correlation between gill score and gross pathology was recorded in the naïve smolt during the infection trial. This may be an indication of the differences between initial and subsequent infections (as the fish in the field trial had undergone previous cycles of AGD and freshwater treatment) and may also reflect differences in host tolerance or resistance to amoeba exposure between naïve smolts and larger fish following several rounds of bathing and reinfection.

Taking into account the additional factors assessed for each of the assays, it is clear that the SYBR® Green protocols are generally cheaper to run as they do not require the inclusion of costly hydrolysis probes. The higher cost of the commercial product is due to the inclusion of several other reagents, required for controls and standard curves. In relation to time, the SYBR® Green assays require longer run times due to addition of melt curve analyses which result in a reduced throughput rate in comparison to that of the TaqMan® protocols. Protocol 1 was seen to perform most favorably across most of the attributes assessed and was ranked by median score as the most suitable assay. Following this it was decided to compare protocol 1 with traditional screening methods (gross gill scoring and histopathology) during an infection trial.

The gross gill scoring, histological scoring and in particular the molecular data presented showed that detection of *N. perurans* was possible within two weeks post-infection and has been previously reported (Morrison et al., 2004; Taylor et al., 2007). Histological examination of the samples in this study identified pathological changes within the first week; however the observation of amoeba in the presence of

pathology and therefore case definition was not confirmed by histology until the second week. While histopathology can indicate both the presence of the pathogen and resultant host response, it requires destructive sampling which could potentially limit the scale of epidemiological studies (Adams et al., 2004; Douglas-Helders et al., 2001) and is not suitable for screening valuable fish e.g. tagged individuals in a breeding program. Molecular analysis did however confirm the presence of *N. perurans* in the first 48 hours of infection and subsequently at each further sampling point. This clearly indicates the usefulness of this non-destructive molecular diagnostic assay for the early detection of *N. perurans* and in sub-clinical cases of AGD. Additionally, with significant gains made through selective breeding (as measured by reduced gill score, Kube et al., 2012) there is an opportunity to fully optimise non-destructive sampling techniques in conjunction with molecular methods to help inform management decisions such as when to treat fish and also determine the efficacy of treatments, and to more finely measure potential gains in AGD resistance/resilience.

3.6 Author Contributions

Conceived and designed the experiments: Jamie Downes, Richard Taylor, Mat Cook, Eugene MacCarthy and Neil Ruane. Performed the experiments and sample analysis: Jamie Downes, Megan Rigby, Richard Taylor, Ben Maynard, Mar Marcos-lopez and Evelyn Collins. Writing and editing manuscript: Jamie Downes, Richard Taylor, Eugene MacCarthy, Ian O'Connor, Hamish Rodger, Neil Ruane, and Mat Cook.

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Chapter 4

**Investigation of co-infections with pathogens associated with gill disease in
Atlantic salmon during an amoebic gill disease outbreak**

4.1 Abstract

Gill diseases are a complex and multifactorial challenge for marine farmed Atlantic salmon. Co-infections of putative pathogens are common on farms; however there is a lack of knowledge in relation to potential interactions of these pathogens. The objective of this study was to determine the prevalence and potential effects of a number of these agents, namely *Neoparamoeba perurans*, *Desmozoon lepeophtherii*, *Candidatus Branchiomonas cysticola*, *Tenacibaculum maritimum* and salmon gill pox virus (SGPV) during a longitudinal study on a marine Atlantic salmon farm. Gill samples were collected from stocking until harvest, on which real-time PCR was used to determine the presence and sequential infection patterns of these pathogens. A number of multi-level models were fit to determine the effect of these putative pathogens and their interaction on gill health (measured as gill histopathology score), while adjusting for the effect of water temperature and time since the last freshwater treatment. Results indicate that between week 12 and 16 post-seawater transfer, colonisation of the gills by all pathogens had commenced and by week 16 of production each of the pathogens had been detected. *D. lepeophtherii* and *Candidatus B. cysticola* were by far the most prevalent of the potential pathogens detected during this study. Detections of *Tenacibaculum maritimum* were found to be significantly correlated to temperature showing distinct seasonality. Salmon gill pox virus (SGPV) was found to be highly sporadic and detected in the first sampling point, suggesting a carryover from freshwater stage of production. Finally, the model results indicated no clear interaction effect between any of the pathogens. Additionally, the models showed that the only variable which had a consistent effect on the histology score was *N. perurans*.

4.2 Introduction

Gill disease (GD) is one of the most serious challenges with respect to health and welfare for global marine salmonid culture (Munday et al. 2001; Steinum et al. 2010; Rodger et al. 2011). As gills are in direct contact with the environment, they are particularly susceptible to water borne irritants, environmental changes and infections. Gill disease is generally complex, often multifactorial and highly sporadic (Mitchell et al. 2012). Losses occur through direct mortalities, poor growth rates, and greater conversion rates and can increase vulnerability to other pathogens (Rodger 2007).

While amoebic gill disease (AGD) is perhaps the most significant disease in terms of gill health and economic impact (Steinum et al. 2008, Taylor et al. 2009, Nowak et al. 2013, Rodger 2014, Oldham et al. 2016), there are numerous other agents that are associated with gill disease (Mitchell & Rodger 2011; Gunnarsson et al. 2017). Proliferative gill inflammation (PGI) was the term introduced to describe recurring gill disease in Atlantic salmon *Salmo salar* in Norway with a multifactorial aetiology (Kvellestad et al. 2005, Steinum et al. 2010). This disease has been the cause of significant losses in Norway, with similar pathologies occurring in Scotland and Ireland (Mitchell & Rodger 2011; Matthews et al. 2013)

Desmozoon lepeophtherii (syn. *P. Theridion*) has previously been suggested to play a significant role in PGI (Nylund et al. 2010). The microsporidian has been found in high prevalence regardless of an actual diagnosis of clinical PGI in Norway, however, it was detected at 30 times greater abundance in fish with confirmed outbreaks than in unaffected fish (Steinum et al. 2010; Matthews et al. 2013). Additionally, *Candidatus* Branchimonas cysticola has been identified as the main agent of epitheliocysts in Norway and a relationship has been previously described between the epitheliocyst load and severity of PGI (Toenshoff et al. 2012; Mitchell et al. 2013). Some variation in opinion exists as to whether or not epitheliocystis is of significance, as some findings have been considered to be incidental (Clark & Nowak 1999) and densities of *Ca. B. cysticola* were not significantly different between fish with gill disease and healthy fish (Gunnarsson et al. 2017). However, a number of studies in Norway found gill-related disease attributed to epitheliocystis (Nylund et al. 1998; Kvellestad et al. 2005; Steinum et

al. 2010). Some observations have found mortalities of up to 80% due to PGI which had associated epitheliocystis (Mitchell & Rodger 2011).

Tenacibaculum maritimum is the causative agent of tenacibaculosis an ulcerative disease in marine fish (Mitchell & Rodger 2011; Fringuelli et al. 2012), with associated gill lesions being described first in chinook salmon *Oncorhynchus tshawytscha* (Chen et al. 1995). Juvenile fish and temperatures above 15°C have been identified as risk factors (Toranzo et al. 2005), with Atlantic salmon found to be particularly susceptible (Soltani et al. 1996). Transmission of the bacterium through sea water and directly from host to host, as well as the involvement of jellyfish have been proposed as possible routes of infection (Ferguson et al. 2010; Mitchell & Rodger 2011; Fringuelli et al. 2012)

Salmon gill poxvirus (SGPV) was detected in the gills of salmon suffering from proliferative gill disease (PGD) in both freshwater and marine sites, with farms suffering high losses associated with the presence of the virus (Nylund et al. 2008). In areas of the gill where a large proportion of the poxvirus was found, proliferation of the gill epithelial cells and invasion of inflammatory cells was observed with 20% and 80 % mortality in freshwater and marine sites respectively (Nylund et al. 2008). However, at the marine site it was recorded that a *Neoparamoeba sp.* was also present which may have contributed to the mortality, furthermore, the impact of this virus as a primary pathogen remains undetermined (Steinum et al. 2008; Mitchell & Rodger 2011; Gjessing et al. 2015). These further highlights the multifactorial nature of GD and the potential interactions between agents detected in the gill.

The majority of field studies have generally investigated AGD and other gill diseases in isolation, however, it has been well documented that co-infections are a common occurrence and this is an issue that has received limited scrutiny in aquatic animals (Kotob et al. 2016). While all these agents may contribute to gill pathology, it is still not clear whether many of these agents are primary or secondary invaders. Additionally there is also a lack of understanding in relation to interactions between these agents (Oldham et al. 2016; Gunnarsson et al. 2017)

This study utilised gill samples collected during a longitudinal study in a marine Atlantic salmon farm over a full production cycle from 2013 to 2014 in Ireland (Downes et al. 2015). Pathogen prevalence was determined by real-time PCR in conjunction with histopathology. Several multi-level models were fitted to determine

whether these putative pathogens had an effect on pathology or if there was any interaction between them.

4.3 Methods

4.3.1 Samples

The samples were collected as part of a longitudinal study investigating AGD on a site in the southwest of Ireland from the period beginning May 2013 to September 2014 (Downes et al. 2015). At each sampling point 5 feeding fish were selected from 2 fixed cages on site (n=10) using a hand net. The second gill arch on the right hand side was excised from each fish and placed into 1 ml RNA later (Sigma) for molecular analysis. Total DNA and total RNA was extracted from 25mg of gill filaments using DNA mini kit (Qiagen) and RNeasy mini kit (Qiagen) respectively, according to manufacturer's instructions for animal tissue. The eluted DNA was stored at -20°C and the RNA was held at -80°C. The second gill arch on the left hand side was excised from each fish and immediately fixed in 10% neutral buffered formalin for histological processing. Sections (5µm) from paraffin-embedded gill samples were stained with haematoxylin and eosin, and examined on an Olympus BX51 microscope. The scoring of the pathology was based on the method developed by Mitchell et al. (2012) as per Downes et al. (2015).

4.3.2 Environmental and Farm data

Temperature data was obtained using StowAway® Tidbit™ sensors which were attached to a cage pontoon on site. Sensors were placed at the depth of 10m and logged on an hourly basis as part of the Marine Institute Temperature Monitoring Programme (www.marine.ie/home/publicationsdata/data/IMOSIMOSTidbit.htm). Information on mortalities, freshwater treatments and fish weight over time were provided by the site manager.

4.3.3 Molecular analysis

All samples were tested for *N. perurans*, *D. lepeophtherii*, *Can. B. cysticola*, *T. maritimum* and salmon gill pox virus. The assays (Table 13) were run as described

using an Applied Biosystems AB7500 real-time instrument and associated software. Each run consisted of 45 cycles and included a positive control and a negative control. For every 20 samples an internal process control, IPC (Internal process control, Life technologies) and external process control, salmonid elongation factor-1 α (Bruno et al. 2007) were used. The threshold at which samples were considered to be positive was maintained for each target across all runs. A Ct value of 45 was assigned to negative samples so they could be included in analysis. In order to present the results in an intuitive manner Ct values were converted to a gill load index (45-observed Ct value) for each putative pathogen.

Table 13 Targets and assays used for molecular analysis during this study

| Target | Primer/Probe | Sequence (5' - 3') | Reference |
|-------------------------|--------------|-----------------------------------|------------------------|
| <i>T. maritimum</i> | Mar 4 For | TGCCTTCTACAGAGGGATAGCC | Fringuelli et al. 2012 |
| | Mar reverse | CTATCGTTGCCATGGTAAGCCG | |
| | Mar probe | CACTTTGGAATGGCATCG | |
| <i>N. perurans</i> | NP1 | AAAAGACCATGCGATTTCGTAAAGT | Downes et al. 2015 |
| | NP2 | CATTCTTTTCGGAGAGTGGAAATT | |
| | NPP | ATCATGATTCACCATATGTT-MGB | |
| <i>D. lepeophtherii</i> | Nuc For | CGGACAGGGAGCATGGTATAG | Nylund et al. 2010 |
| | Nuc Rev | GGTCCAGGTTGGGTCTTGAG | |
| | Nuc probe | TTGGCGAAGAATGAAA | |
| Salmon Gill Poxvirus | For | ATCCAAAATACGGAACATAAGCAAT | Gjessing et al. 2015 |
| | Rev | CAACGACAAGGAGATCAACGC | |
| | Probe | CTCAGAAACTTCAAAGGA | |
| <i>Ca. B. cysticola</i> | BPr2 | AAT ACA TCG GAA CGT GTC TAG TG | Mitchell et al. 2013 |
| | BPr2 | GCC ATC AGC CGC TCA TGT G | |
| | BPr2 (Probe) | CTC GGT CCC AGG CTT TCC TCT CCC A | |
| PRV | For | TGC TAA CAC TCC AGG AGT CAT TG | Løvoll et al. 2012 |
| | Rev | TGA ATC CGC TGC AGA TGA GTA | |
| | Probe | CGC CGG TAG CTC T | |

4.3.4 Model analysis

To model the gill histopathology score of each sampled fish (N fish in total), which is an ordinal quantity going from 0 to 3 (4 levels), an ordered logistic model was used, as detailed below;

$$\log\left(\frac{P(y_i \leq k)}{1 - P(y_i \leq k)}\right) = \alpha_k - \phi_i$$

$$\phi_i = \gamma_w + \beta X_i \quad (1)$$

where $\log\left(\frac{P(y_i \leq k)}{1 - P(y_i \leq k)}\right)$ is the log-cumulative-odds of observing in fish i a gill score, y_i , less than or equal to k , with $i = 1, \dots, N$ and $k = 0, 1, 2$. In this model the log-cumulative-odds of each gill score k are defined as the sum of an intercept α_k and a linear model, ϕ_i , where γ_w is the varying effect of the week of sampling w (with $w = 1, \dots, 26$), β is the vector of population effects and X_i is the vector of

covariates for fish i . Regarding the interpretation of the regression coefficients of the linear model, a $\beta > 0$ would indicate that an increase in the predictor variable is associated with an increase in the mean gill histopathology score.

The week effect - γ_w , was modelled as a Gaussian process, with a multivariate prior of the form

$$\gamma \sim MVNormal([0, \dots, 0]_{26}, K) \quad (2)$$

with covariance matrix K . The covariance between any pair of weeks i and j was estimated using the squared exponential covariance function of the form

$$K_{i,j} = \eta^2 \exp(-\rho^2 D^2) + \delta_{i,j} \sigma^2 \quad (3)$$

where η , ρ , σ are hyperparameters defining the covariance function. η - the scale factor, is the maximum covariance between any two weeks i and j , ρ - the (inverse) length scale, determines the rate of decline of the covariance, D is the Euclidean distance between weeks i and j , $\delta_{i,j}$ is the Kronecker function with value 1 if $i = j$ and 0 otherwise, σ is the covariance when $i = j$, the noise variance (Rasmussen and Williams, 2006; McElreath, 2016b; Stan Development Team, 2016b).

The variables evaluated for their association with gill score were the Ct values of the measured agents. Their estimated effect was adjusted for water temperature, and time since the last freshwater treatment. Ten observations (5 from each cage) were removed from the data set, as these lacked gill score data during week 50 of follow up.

All variables were scaled to have a mean of 0 and standard deviation of 0.5. These transformations allow for the interpretation of the regression coefficients to be more transparent, by making them directly comparable from a parameter estimates table (Gelman, 2008), although plotting is a much clearer means of understanding the predictor variables' effects, and hence the main approach used here. In addition, Ct values of *N. perurans* and putative pathogens were multiplied by -1 in order to make these values intuitively reflective of higher concentration in the gills (i.e. higher values mean higher concentration of these agents).

Model fitting was carried out in a Bayesian framework, with priors

$$\alpha \sim Normal(0, 5) \quad (4)$$

$$\beta \sim Normal(0, 0.5)$$

For the covariance matrix the priors used were

$$\eta^2 \sim HalfCauchy(0, 1) \quad (5)$$

$$\rho^2 \sim \text{HalfCauchy}(0, 1)$$

$$\sigma^2 \sim \text{HalfCauchy}(0, 1)$$

Nine multi-level models were fit to determine the effect of these putative pathogens. These models were: 1) a null model with no covariates in it, 2) a model with only water temperature and time since the last freshwater treatment, a model with only the Ct values of *N. perurans*, adjusting for water temperature and time since the last freshwater treatment, 3) a model with the Ct values of all the putative gill pathogens and their interaction with *N. perurans* (full model), adjusting for water temperature and time since the last freshwater treatment, 4) a model with the Ct values of all the putative gill pathogens assuming no interaction with *N. perurans* (main effects model), adjusting for water temperature and time since the last freshwater treatment, and 5-9) 4 models evaluating the effect of the Ct values of each putative gill pathogen, adjusting for *N. perurans*, water temperature, and time since the last freshwater treatment.

Each model was initially fitted using 4 chains of 4,000 iterations with a warm-up of 2,000 iterations for assessing model convergence, after which an individual chain of 20,000 iterations with warm-up of 4,000 iterations was used for inference for each model. Model convergence diagnostics included visual checking of trace plots, to visually evaluate stationarity and mixing of the chains, Gelman-Rubin convergence diagnostic, \hat{R} , and the number of effective samples (Gelman and Rubin, 1992; Gelman et al., 2014).

Models were fit using Stan's Hamiltonian Monte Carlo sampling (Stan Development Team, 2016b) in the R statistical environment (R Development Core Team, 2017), using the rstan package (Stan Development Team, 2016a). Model predictive accuracy was evaluated using Leave-one-out information criterion with the loo package in R (Vehtari et al., 2016).

Finally, posterior predictive checks were used to evaluate if simulated samples from the model matched the original data. For this 16,000 random samples from an ordered categorical probability distribution, with the estimated parameters, were generated for each observation, using the package rethinking (McElreath, 2016a). Evaluation of agreement between simulated and observed data was done visually through histograms, using the package bayesplot (Gabry, 2017).

4.4 Results

4.4.1 Environmental and Farm data

During the production cycle there was an overall mortality of 33%, with three distinct peaks in mortality occurring during production week 17 (2/8/2013) with a max of 2.02% mortality, week 26 with 3.00% (4/10/13) and week 34 (29/11/13) where 2.43% mortality was recorded. Gill disease, more specifically AGD, was attributed to an estimated 18% of the total mortality on site through the production cycle. Temperature peaked at 17.9°C in week 16 (26/7/2013) and declined to 8.4°C week 45 (14/02/2014). In total, there were 6 freshwater bath treatments, with the first 3 requiring the entire site to be treated and the subsequent 3 saw only selected cages treated.

4.4.2 Histology

Histopathology scores were recorded over the full range of the scoring method following week 12 each subsequent sampling point had at least some mild form of pathology. Hyperplasia, lamellar fusion and vesicle formation were the most commonly observed pathology during the production cycle. In addition to the pathology described, amoebae were observed on a regular basis (Fig. 13A). Epitheliocystis was recorded in a number of samples through the study (Fig. 13 B) but did not contribute particularly to the overall pathology recorded. Pathology characteristic of pox-virus or related to *D. lepeophtherii* and *T. maritimum* was not observed in the samples.

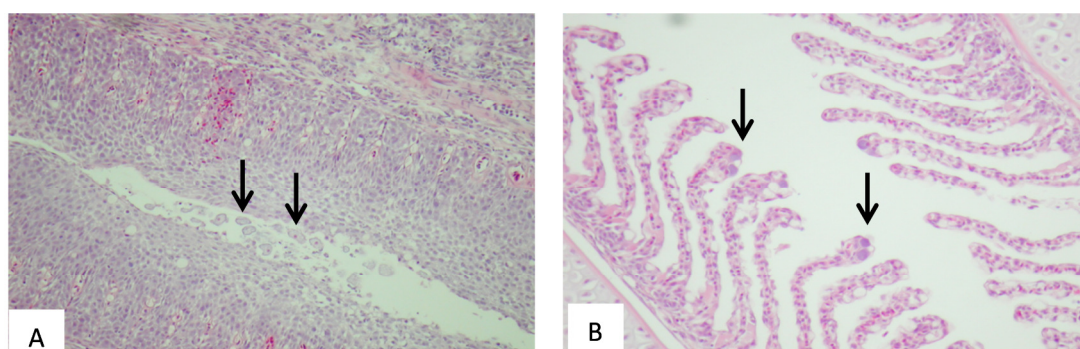


Figure 13 (A) Severe Hyperplasia and fusion with associated amoeba (Arrows), (B) Epitheliocystis(Arrows)

4.4.3 Molecular analysis

4.4.3.1 *Neoparamoeba perurans*

In the 4th week (7/5/2013) of production *N. perurans* was initially detected with 30% of the fish testing positive (Table 14), however, it was not until the period between week 12 (28/6/13) and 16 (31/7/16) that there was a further proliferation of the amoeba, where 100% of samples were positive. The detections of *N. perurans* varied considerably due to treatments using freshwater baths. The average gill load for *N. perurans* increased on several occasions with the greatest peak as early as week 16 with a gill load index of 12.67 (+/-0.57) (Fig. 14 A).

4.4.3.2 *Desmozoon lepeophtherii*

Desmozoon lepeophtherii was initially detected during production week 12 (28/6/13) and by week 16 (26/07/13) there was 100% prevalence until week 41 (17/01/13) when some variation in the amount of samples that were positive was observed; however, this never fell below 80% (Table 14). The average gill load observed for *D. lepeophtherii* peaked at week 24 (20/9/13) with gill load index = 20.64 (+/-0.49), the latter value gradually declined over the remainder of the production cycle to a gill load index of 10.72 (+/-1.57) at the final sampling point (Fig. 14B).

4.4.3.3 *Candidatus Branchiomonas cysticola*

The first detection of *Ca. B. Cysticola* occurred during production week 16 (26/07/2013) where 20% of the samples were positive and by week 28 (18/10/2013) 100% of samples were positive. Following this, there was very little variation with just 3 other sample points in which negative samples were observed - weeks, 30, 62 and 67 (Fig. 14C). The average gill load for *Ca. B. Cysticola* peaked at week 36 with a value of 19.15 (+/-0.55) and gradually decline until the final sample point with an average gill load of 9.99 (+/-1.37) (Fig. 14C).

4.4.3.4 *Tenacibaculum maritimum*

Detection of *T. maritimum* first occurred on production week 13 (5/7/2013), with detections peaking during week 24 where 100% of samples were positive; however, the prevalence declined in the subsequent period from week 28 (18/10/16) (Table 14), Furthermore during week 61 (6/6/2014) a noticeable increase in the percentage positive for *T. maritimum* was observed again. The average gill load of *T. maritimum* was recorded as having two peaks during the weeks 24 (20/9/2013) and 67 (18/7/2014) with values of 10.91 (+/-0.93) and 8.97 (+/-1.65), respectively (Fig. 14D). There was a significant correlation ($r=0.48$, $p<0.05$) between the prevalence of *T. Maritimum* and temperature.

4.4.3.5 Salmon gill pox virus

Salmon gill pox virus was detected at the first sampling point during week 3 and subsequently detected sporadically throughout the remainder of the production cycle. There was just a single sample point with 100% of the samples positive for SGPV which was during week 32 (15/11/13) (Table 14). This peak in prevalence coincided with the highest average gill load index of 13.14 (+/-1.19) (Fig. 14E).

Table 14 Percentage of samples positive for pathogens throughout the production cycle from April 2013 to July 2014 (AGD-amoebic gill disease, *Desmozoon lepeophtherii*, *Candidatus Branchiomonas cysticola*, *Tenacibaculum maritimum*, Salmon gill pox virus)

| Date | week no | AGD | <i>D. lepeophtherii</i> | <i>Ca. B. cysticola</i> | <i>T. maritimum</i> | PRV | SGPV |
|-----------|---------|-----|-------------------------|-------------------------|---------------------|-----|------|
| 26-Apr-13 | 3 | 0 | 0 | 0 | 0 | 0 | 70 |
| 07-May-13 | 4 | 30 | 0 | 0 | 0 | 0 | 30 |
| 23-May-13 | 6 | 0 | 0 | 0 | 0 | 0 | 0 |
| 05-Jun-13 | 8 | 0 | 0 | 0 | 0 | 0 | 0 |
| 11-Jun-13 | 9 | 0 | 0 | 0 | 0 | 0 | 0 |
| 28-Jun-13 | 13 | 10 | 40 | 0 | 0 | 20 | 0 |
| 09-Jul-13 | 14 | 40 | 90 | 0 | 70 | 80 | 0 |
| 31-Jul-13 | 16 | 100 | 100 | 20 | 60 | 40 | 0 |
| 16-Aug-13 | 19 | 10 | 100 | 20 | 90 | 100 | 30 |
| 02-Sep-13 | 21 | 60 | 100 | 40 | 90 | 40 | 0 |
| 25-Sep-13 | 24 | 80 | 100 | 90 | 100 | 100 | 40 |
| 18-Oct-13 | 28 | 30 | 100 | 100 | 60 | 90 | 10 |
| 04-Nov-13 | 30 | 90 | 100 | 80 | 90 | 100 | 20 |
| 18-Nov-13 | 32 | 100 | 100 | 100 | 50 | 100 | 100 |
| 29-Nov-13 | 34 | 90 | 100 | 100 | 30 | 100 | 10 |
| 17-Dec-13 | 36 | 80 | 100 | 100 | 50 | 100 | 45 |
| 17-Jan-14 | 41 | 60 | 90 | 100 | 40 | 100 | 45 |
| 07-Feb-14 | 44 | 10 | 100 | 100 | 10 | 100 | 10 |
| 13-Mar-14 | 49 | 30 | 100 | 100 | 10 | 100 | 40 |
| 26-Mar-14 | 50 | 10 | 85 | 100 | 10 | 80 | 15 |
| 08-Apr-14 | 52 | 90 | 100 | 100 | 10 | 70 | 10 |
| 24-Apr-14 | 55 | 30 | 95 | 100 | 35 | 80 | 10 |
| 06-May-14 | 56 | 10 | 80 | 100 | 0 | 90 | 0 |
| 03-Jun-14 | 61 | 100 | 100 | 100 | 60 | 100 | 0 |
| 09-Jun-14 | 62 | 66 | 85 | 95 | 40 | 95 | 0 |
| 30-Jun-14 | 65 | 100 | 80 | 100 | 60 | 60 | 33 |
| 17-Jul-14 | 67 | 40 | 90 | 90 | 80 | 80 | 40 |

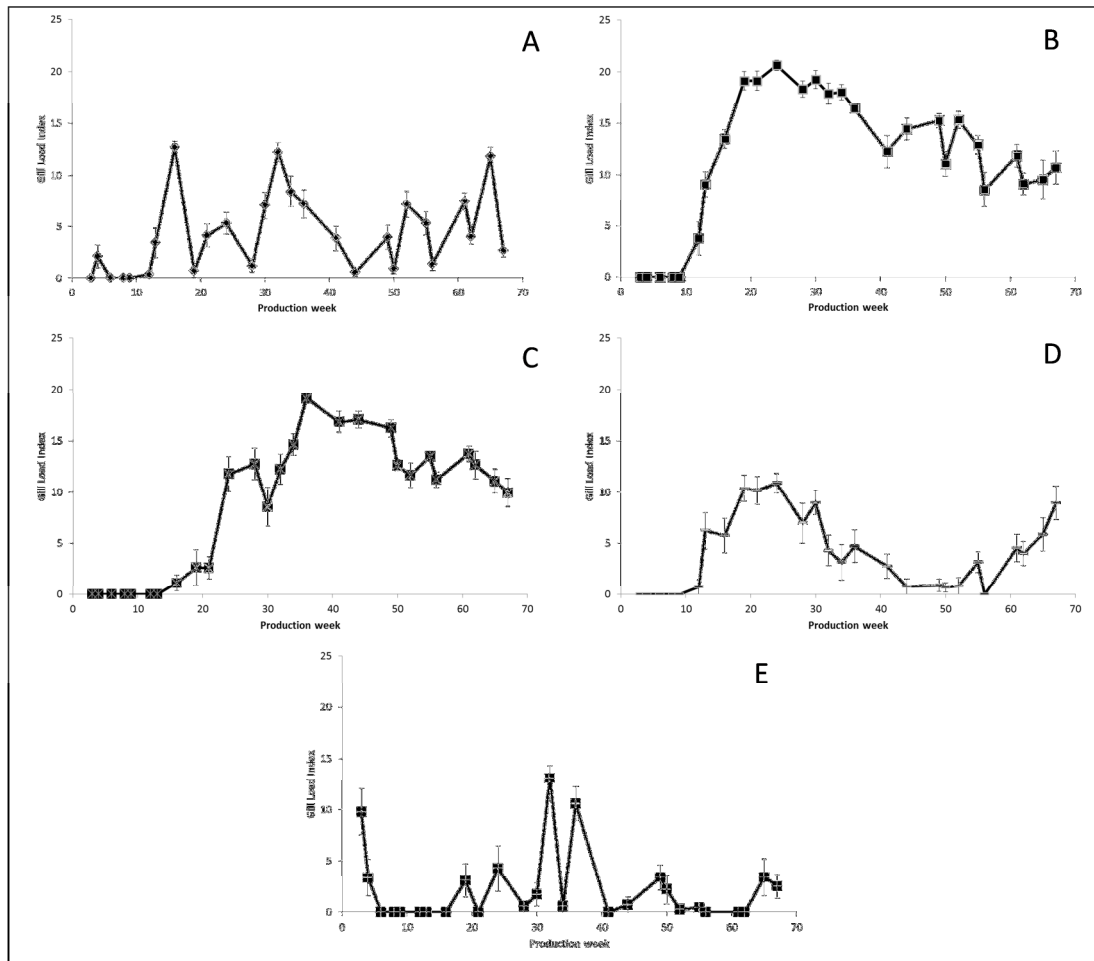


Figure 14 Average gill load index for (A) *N. perurans*, (B) *D. lepeophtherii*, (C) *Ca. B. cysticola*, (D) *T. maritimum*, (E) Salmon gill pox virus over the entire production cycle.

4.4.4 Model analysis

Model comparisons are presented in Table 15. The best ranking model was the model including *N. perurans* and *Ca. B. cysticola*, adjusted for water temperature and time since the last freshwater treatment (leave-one-out cross-validation information criteria, looic = 614.48), indicating that these are the only putative pathogens showing a meaningful association with the gill histopathology score. The full model was the lowest ranking model (looic = 619.35), preceded by the model including only temperature and time since the last freshwater treatment (looic = 618.66) and the null model (looic = 618.48). This indicates that addition of the other gill pathogens and their interaction with *N. perurans* does not increase the model's predictive accuracy. Further exploration of the full model shows that besides the effect of *N. perurans* (the entire 95% probability interval of its estimated effect is above 0), the other variables included in the model could be either negatively or

positively associated with a higher gill score (Fig.15). A potential exception is the estimated effect of *Ca. B. cysticola* which, although crossing the null value of zero, had most of its posterior distribution below this value. This potential effect is also shown in the top-ranking model, which includes both predictors. Parameter estimates from this model are presented in Table 16. This table shows that for a decrease of two SD in the Ct for *N. perurans* (a decrease of 9.58 Ct's) the odds of having a higher gill score increase by 2.1 (95% PI: 1.20 – 3.63). It also shows a potential protective effect of *Ca. B. cysticola*, where a decrease of two SD (7.02 Ct's) makes the odds of higher gill score decrease by 17% (odds ratio of 0.83), although the evidence for this effect is not strong (95% PI of 0.42 – 1.60).

Table 15 Model comparisons for gill histopathology score using leave-one-out information criterion (looic) (NP – *N. perurans*; BC- *Ca. B. cysticola*; DL- *D. lepeophtherii*; PV- salmon gill pox virus; TM - *T. Maritimum*; FW- freshwater bath)

| Model* | looic | se_looic | p_loo | se_p_loo |
|--------------|--------|----------|-------|----------|
| NP + BC | 614.48 | 23.2 | 25.31 | 1.50 |
| NP | 614.52 | 23.21 | 24.76 | 1.46 |
| NP + PV | 615.51 | 23.22 | 25.32 | 1.48 |
| NP + TM | 615.76 | 23.24 | 25.44 | 1.51 |
| NP + DL | 615.78 | 23.22 | 25.19 | 1.47 |
| Main effects | 617.93 | 23.37 | 27.04 | 1.58 |
| Null | 618.48 | 22.7 | 24.69 | 1.44 |
| Temp + FW | 618.66 | 22.68 | 24.76 | 1.45 |
| Full | 619.35 | 23.52 | 29.18 | 1.72 |

Table 16. Parameter estimate's posterior distribution of gill score top ranked model

| Parameter | Parameter estimates | | | | Cumulative odds ratio | | |
|-------------------------|---------------------|------|-------|-------|-----------------------|------|-------|
| | mean | SD | 2.5% | 97.5% | mean | 2.5% | 97.5% |
| α_0 | -1.83 | 0.44 | -2.72 | -0.97 | 0.16 | 0.07 | 0.38 |
| α_1 | 1.40 | 0.43 | 0.55 | 2.28 | 4.06 | 1.73 | 9.78 |
| α_2 | 2.78 | 0.46 | 1.88 | 3.70 | 16.12 | 6.55 | 40.45 |
| Temperature | 0.24 | 0.42 | -0.59 | 1.07 | 1.27 | 0.55 | 2.92 |
| Time since FW | 0.30 | 0.43 | -0.56 | 1.12 | 1.35 | 0.57 | 3.06 |
| <i>N. perurans</i> | 0.74 | 0.28 | 0.18 | 1.29 | 2.10 | 1.20 | 3.63 |
| <i>Ca. B. cysticola</i> | -0.19 | 0.34 | -0.86 | 0.47 | 0.83 | 0.42 | 1.60 |

α_0 , α_1 , and α_2 : baseline cumulative log-odds (odds ratio) of a gill score of 0, 1, and 2, respectively. Time since FW: time since the last freshwater treatment

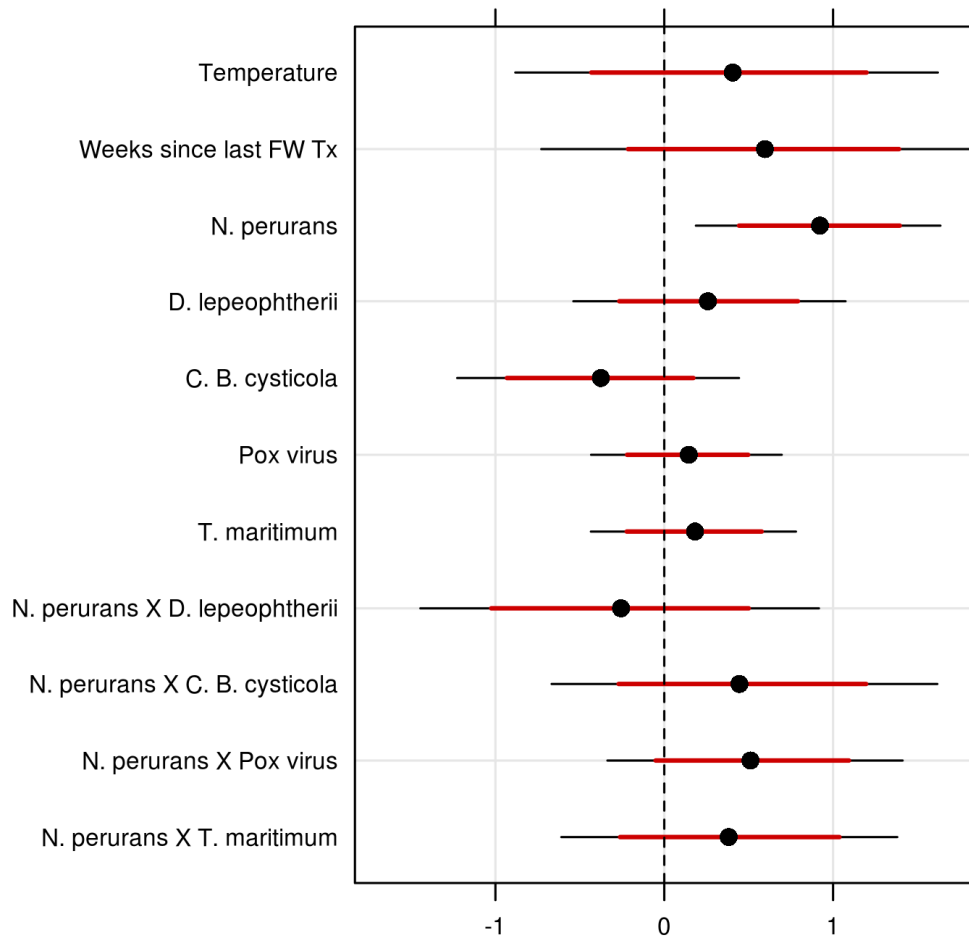


Figure 15. Parameter estimates of population level effects for the full model. Black dot: median, red thick line: 80% probability interval, black thin line: 95% probability interval.

Distributions of simulated samples from the top-ranking model (NP + *Ca. B. cysticola*) are presented in Fig. 16. Here, it can be seen that there is a good agreement between observed data and simulated observations from the model's posterior distribution. This way, the observed (simulated mean and 95% PI) proportions of gill scores of 0, 1, 2, and 3 were 0.217 (0.216, 0.163 – 0.270), 0.483 (0.480, 0.401 – 0.553), 0.160 (0.164, 0.110 – 0.220), and 0.140 (0.140, 0.093 – 0.190), respectively. These results indicate that the model chosen to fit this data is adequate.

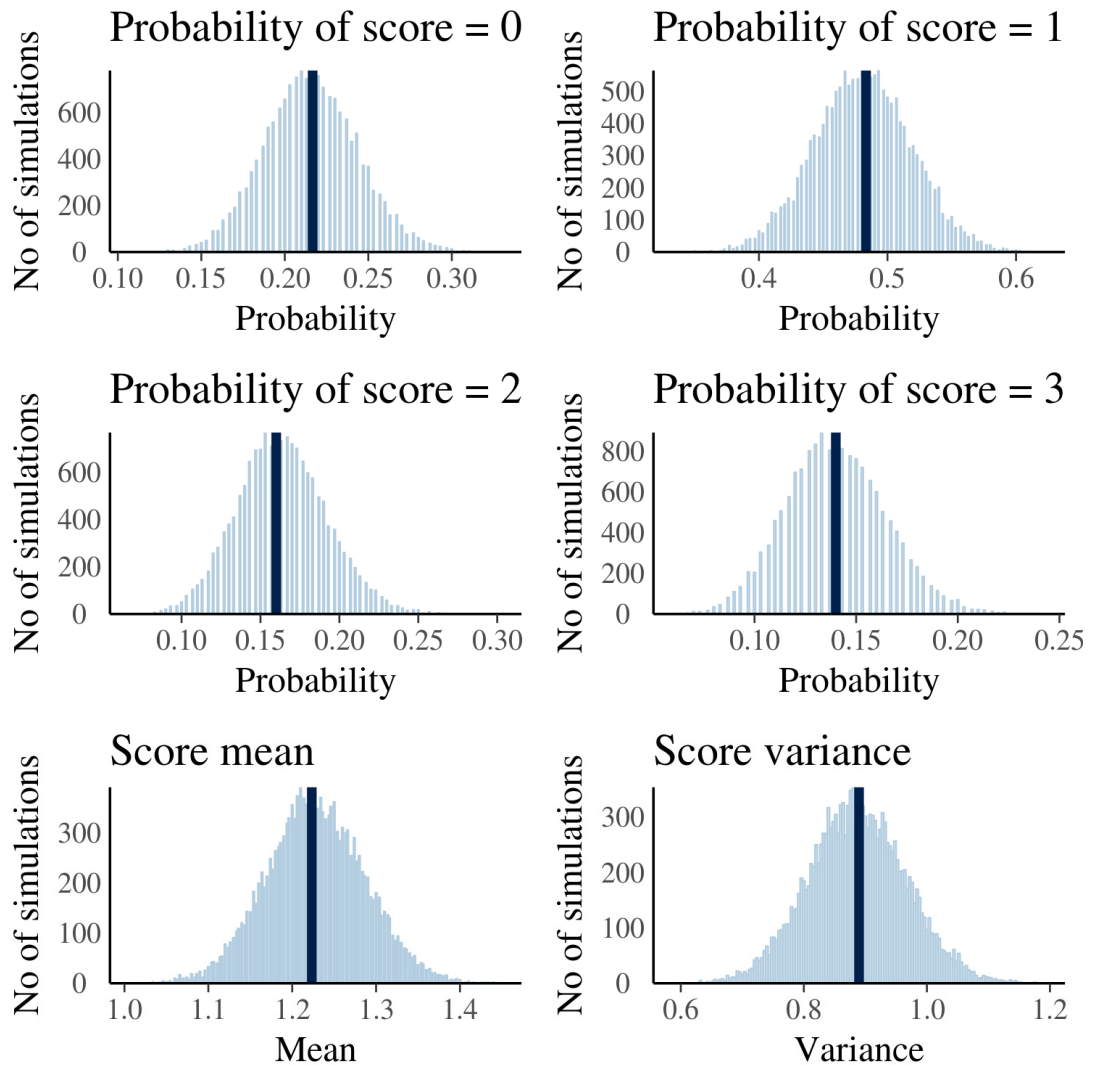


Figure 16. Posterior predictive checks of the top ranked gill score model. Observed (dark blue line) and simulated (light blue lines) sample statistics

4.5 Discussion

Recently there has been an increased focus on the multifactorial aspect of gill disease (Gunnarsson et al. 2017; Gjessing et al. 2017) as numerous putative pathogens have been identified and found to be connected with gill disease (Steinum et al. 2010; Mitchell & Rodger 2011). There is a lack of knowledge in relation to the occurrence and development of co-infections with many of these putative pathogens (Oldham et al. 2016). This study was undertaken to determine the prevalence of gill disease-associated pathogens during a longitudinal study investigating AGD in Ireland (Downes et al. 2015). The intent was to elicit if or any interactions between

these pathogens occurred during an outbreak using a suite of molecular diagnostic assays, histopathology and site information.

The pathogens included in this study, deemed to have the potential to interact or influence gill disease, were detected throughout the production cycle with varying levels of prevalence. Each of the putative pathogens was detected by week 16 (31/7/13) of the production cycle. Proliferation of these putative pathogens on the gills generally occurred 12 to 16 weeks' post sea water transfer. Following this period all pathogens apart from SGPV increased in prevalence over a relatively short period. Prior to the ~12-week mark only *N. perurans* and SGPV were detected in the samples. This data suggests that this is an extremely important period of production during which the intensification of gill screening and monitoring would be warranted. This period for S1 smolts coincides with the onset of the summer months where observed temperatures began to increase significantly. This creates additional environmental pressures on potentially compromised stocks in addition to perhaps influencing the growth or proliferation of the pathogens.

In a previous publication on the AGD outbreak that occurred on this site (Downes et al. 2015), the prevalence of *N. perurans* was discussed. The prevalence of *N. perurans* was heavily influenced and controlled by freshwater treatments. These treatments proved to be an effective method to reduce the number of PCR-positive fish immediately following treatments. There were three peaks in mortality associated with AGD during this production cycle. In the second year of production there was a continued high prevalence of *N. perurans* detected without any associated mortality. This pattern has continued to be observed on sites in Ireland containing S1's smolts in subsequent years (unpublished data).

D. lepeophtherii and *Ca. B. Cysticola* were by far the most prevalent of the putative pathogens detected during this study. Once established in the sample population, there was relatively little variation in their prevalence (*D. lepeophtherii* 80 to 100% and *Ca. B. Cysticola* 80 to 100%). This is similar to the prevalence recorded in a study in Norway by Gunnarsson et al. (2017) where they showed high prevalence for both *D. lepeophtherii* (100%) and *Ca. B. Cysticola* (83 to 100%). The pattern of infection in relation to *D. lepeophtherii* corresponds with the infection pattern observed by Sveen et al. (2012), wherein, the gill load of *D. lepeophtherii* was highest in autumn followed by a decline over the winter months. In this study, the decline in gill load continues through the next summer until the end of the

sampling. This parasite is frequently detected in high numbers in both healthy gills and gills displaying PGI-like pathology (Mitchell & Rodger 2011). Where populations of fish were diagnosed with gill disease, observations of *D. lepeophtherii* have been 4 fold higher (Gunnarsson et al. 2017) and 30+ times higher (Steinum et al. 2010) than that of fish without a gill disease diagnosis. Co-infections with other agents, opportunistic parasitism or potentially affecting the host's immune system have been suggested as means by which *D. lepeoththerii* may influence PGI (Nylund et al. 2010; Mitchell & Rodger 2011).

The pattern of colonisation of the gills by *Ca. B. cysticola* during this study appears to be slower and more gradual than the other agents. The peak in gill load was not recorded until week 36 (17/12/13) where temperature had fallen to 10°C. High prevalence of *Ca. B. cysticola* has been recorded in a number of studies screening for gill disease (Mitchell et al. 2013; Gunnarsson et al. 2017; Gjessing et al. 2017) and this study concurs with those findings. Once established in the population the prevalence remained high throughout the entire study period. The peak in prevalence and gill load during the winter months would suggest that seasonality or temperature does not have much influence on the prevalence of *Ca. B. cysticola*. The bacteria is commonly associated with epitheliocysts in Atlantic salmon (Toenshoff et al. 2012), indeed epitheliocysts were recorded in the histology of a number of samples in this study. However, epitheliocysts did not contribute to the overall level of pathology to a great extent. It has been noted that such infections are frequently described without any associated pathology or clinical signs (Mitchell & Rodger 2011). The results recorded during this study in addition to a number of other studies advocate that pathological changes, which potentially occur due to *D. lepeophtherii* and *Ca. B. cysticola* are dose-dependent (Steinum et al. 2010, Nylund et al. 2011, Mitchell et al. 2013, Gunnarsson et al. 2017), however such a threshold has yet to be determined.

Levels of *T. maritimum* were significantly correlated ($P>0.05$) to temperature and were clearly demonstrated to show distinct seasonality. An increase in prevalence was observed in the Summer/Autumn of the first year of production. This was followed by a decline in the percentage of positive fish over the winter months. As temperatures began to increase the following year, prevalence of *T. maritimum* also began to return. Higher temperatures (above 15°C) and salinities have been reported as risk factors for tenacibaculosis (Avendano-Herrera et al 2006). An

experimental study has previously suggested that an underlying AGD outbreak does not predispose fish to *T. maritimum* infections and vice versa (Powell et al. 2005), which correspond with the results observed in this study. During week 26 of production there was mortality recorded due to *Pelagia noctiluca*, a species of jellyfish, which has been reported as a vector for *T. maritimum* (Ferguson et al. 2010). However, *T. maritimum* was established in the population prior to the observed jellyfish bloom. Manipulation of temperature and/or salinity below 10g l⁻¹ have been suggested to reduce morbidity in salmonids infected with *T. maritimum* (Soltani & Burke 1995). However, in this study the presence of the bacterium were not obviously affected by freshwater treatments. The potential importance of *T. maritimum* in salmon culture is further highlighted since the isolation of this bacterium from lumpsucker, which have become increasingly important as a non-medicinal means for sea lice control (Smage et al. 2016).

The detection of salmon gill pox virus was variable throughout the production cycle, with just one sample point had all samples positive (week 32, 18/11/13). Unlike the other pathogens in this study, SGPV was detected in the first sample point 3 weeks post sea-water transfer. This may suggest that there was carryover of the virus from freshwater sources. In fact SGPV has previously been recorded in both the freshwater and marine stage of production in Norway (Gjessing et al. 2015). From the results observed there does not appear to be any discernible pattern/proliferation of infection in comparison to that of the other pathogens screened in this study. Previously cases of combined outbreaks of AGD and SGPV in Norway were attributed to have caused 82% mortality (Steinum et al. 2008, Gjessing et al. 2015). SGPV and *Ca. B. cysticola* have also been shown to horizontally transmit between fish (Wiik-Nielsen et al. 2017)

The results from the multi-level models demonstrated that *N. perurans* is the only pathogen which had a clear effect on the status of the gill histopathology score. Importantly, there did not appear to be any clear effect or interaction with the other putative pathogens included in the models. One potential exception to this was *Ca. B. cysticola*, whose regression coefficient in the top ranking model was observed as being below the null value of zero. This potentially indicates a protective effect, where an increase in the level of *Ca. B. cysticola* would mean a decrease in the gill histopathology score. However, as some of the 95% probability interval was above the null value of zero (28.5% of the posterior distribution), this effect is not strongly

supported by the data (unlike *N. perurans*, where 99.5% of the posterior distribution lied above 0). Hence, this estimated effect of *Ca. B. cysticola* might well be due to random variation in the sampled fish related with the small sample size. Further multi-site studies with a larger sample size, could help further elucidate the effect of this and other putative pathogens.

It should also be noted that freshwater treatments had no influence over the levels of any of the agents other than *N. perurans*. The results of this study would suggest that many of these putative pathogens are part of the autochthonous populations of the fish gills and that these fish may be asymptomatic carriers with the potential for pathological changes given the correct conditions. Furthermore, it is clear from this study and a number of other studies that these agents occur in high prevalence naturally in the gill (Steinum et al. 2010, Matthews et al. 2013, Mitchell et al. 2013, Gunnarsson et al. 2017) and that they appear to rely on dose dependent levels to cause clinical disease. Despite clear gill pathology during an AGD outbreak, a number of other potential pathogens can and do occur in the gill and in this instance without significantly affecting pathology.

4.6 Author contributions

Conceived and designed the experiments: Jamie Downes, Tadaishi Yatabe-Rodriguez and Neil Ruane. Performed the experiments and sample analysis: Jamie Downes, Mar Marcos-lopez, and Evelyn Collins. Data Analysis: Jamie Downes and Tadaishi Yatabe-Rodriguez. Writing and editing manuscript: Jamie Downes, Tadaishi Yatabe-Rodriguez, Eugene MacCarthy, Ian O'Connor, Hamish Rodger and Neil Ruane.

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Chapter 5

A retrospective investigation of putative pathogens on the gills of Atlantic salmon during the earliest outbreaks of amoebic gill disease in Ireland

5.1 Abstract

Amoebic gill disease (AGD) is a significant challenge for Atlantic salmon, *Salmo salar*, aquaculture globally. In Ireland, AGD was first described in Atlantic salmon culture in 1995 and subsequently reoccurred again in 1997. At the time, the disease was attributed to *Paramoeba* sp. This study aimed to analyse archived formalin-fixed paraffin-embedded (FFPE) Atlantic salmon (*Salmo salar*) gill tissue from the first Irish cases of AGD, in order to determine whether or not *Neoparamoeba perurans* was present. Additionally, the FFPE gill tissues were also analysed by real-time PCR for *Candidatus Brachyomonas cysticola*, *Desmozoon lepeophtherii*, *Tenacibaculum maritimum*, and Salmon gill pox virus, to determine whether these putative pathogens were also present in samples from fish at that time. Through the use of molecular diagnostics, this study confirmed that *N. perurans* was the causative agent of the first recorded outbreaks of AGD in Ireland. Additionally, the study found that a number of other putative gill pathogens have been present in Irish aquaculture for over 20 years.

5.2 Introduction

Gill diseases pose a significant challenge for Atlantic salmon *Salmo salar*, aquaculture globally. The most significant of these is amoebic gill disease (AGD) caused by *Neoparamoeba perurans* (Young et al., 2008). AGD has been endemic in the Australian industry since the 1980s, however, the disease has become a significant problem for European salmon aquaculture since its re-emergence in 2011 with Ireland, Norway, France, Scotland and the Faroe Islands all affected (Rodger, 2014; Oldham et al., 2016).

The first case of AGD in Ireland was described in the autumn of 1995 in S1 Atlantic salmon transferred to sea in the spring of that year, with a total of 10 sites showing pathology and associated amoeba (Palmer et al., 1997; Rodger & McArdle, 1996). The case history of these outbreaks (Palmer et al., 1997; Rodger & McArdle, 1996) reveals the typical clinical signs associated with AGD: lethargy, respiratory distress and congregation at the surface of the water. Gill smears taken at the time revealed high mucus levels as well as numerous amoebae. Of 10 sites with confirmed AGD, two recorded mortality exceeding 10%, while three others had less than 5% mortality, with the remaining sites experiencing no significant mortality (Rodger & McArdle, 1996). Between the years 1995 and 2010, there were sporadic and relatively minor outbreaks of AGD (Rodger 2014). Previous studies in Ireland demonstrated that although *Neoparamoeba sp.* were present on the gills of AGD-affected fish, they did not necessarily correlate with the disease and a number of other amoebae species (*Platyamoeba sp.*, *Nolandella sp.*, *Mayorella sp.*, *Vexillifera sp.*) were commonly found on the gills along with ciliate parasites (Bermingham & Mulcahy, 2006; 2007). However, it must be noted that these studies were conducted before the confirmation of *N. perurans* as the causative agent of AGD (Young et al., 2007; Crosbie et al., 2012) and without the use of species-specific molecular diagnostic tools.

In addition to *N. perurans*, a number of other putative pathogens have been implicated in the development of gill pathologies (Kvellestad et al., 2005; Nylund et al., 2008; Mitchell & Rodger, 2011). These include *Candidatus Branchiomonas cysticola*, a cyst-forming bacterium linked with epitheliocystis and potentially playing a role in proliferative gill inflammation (PGI) (Mitchell et al., 2013). Also associated with PGI is the microsporidian parasite, *Desmozoon lepeophtherii*

(Freeman & Sommerville, 2009; Nylund et al., 2010), which has been identified and detected in the gills of salmon regardless of PGI status. However, higher parasite loads have been recorded in fish with PGI (Steinum et al., 2010; Nylund et al., 2011). *Tenacibaculum maritimum*, the causative agent of tenacibaculosis, has been associated with gill, skin and fin lesions in farmed salmonids and marine fish (Avendaño-Herrera et al. 2006; Olsen et al. 2011). More recently, the salmon gill poxvirus (SPGV) was characterised and found to be associated with gill disease and apoptotic respiratory epithelial cells, detected in a number of cases in Norway dating back to 1995 (Gjessing et al., 2015; 2017).

The aim of this study was to use real-time PCR to analyse archived formalin-fixed paraffin-embedded (FFPE) Atlantic salmon gill tissue from the first Irish cases of AGD in 1995 and 1997, in order to determine whether or not *N. perurans* was present. Samples were also analysed by real-time PCR for *Ca. B. cysticola*, *D. lepeophtherii*, *T. maritimum*, and SGPV, to determine whether these putative pathogens were also present in samples from fish at that time.

5.3 Methods

Archived histology blocks (embedded in wax and stored at room temperature) of gill samples originally collected in October and November of 1995 and 1997, from a salmon production site suffering from gill disease in the west of Ireland, were retrieved for this study. Sections (5µm) were cut and stained (Haematoxylin and Eosin) and examined for the presence of amoeba. Sections from each FFPE block with amoeba present were deparaffinised as follows: 8 (10µm) sections were placed in 2ml plastic tubes, 1ml of xylene was added and each tube was vortexed vigorously for 20-30 seconds and centrifuged at 21,130 g for 2 minutes. This was then followed with an alcohol wash step with 1 ml of 100% ethanol, vortexed vigorously for 20-30 seconds and centrifuged at 14000 rpm for 2 minutes. This alcohol wash step was then repeated to ensure that any residual xylene was removed from the tissue. The 2ml plastic tubes were then opened and incubated at 30°C for 30 minutes to allow all residual alcohol to evaporate. Extractions were performed using a QIAamp DNA mini kit (Qiagen) according to manufacturer's instructions for animal tissue. Real-time PCR analyses were performed for the detection of *N. perurans* (Downes et al., 2015), *Ca. B. cysticola* (Mitchell et al., 2013), *D.*

lepeophtherii (Nylund et al., 2010), *T. maritimum* (Fringuelli et al., 2012) and SGPV (Gjessing et al., 2015), using an Applied Biosystems 7500 Real-time PCR system and associated software. All samples were tested in triplicate along with positive controls, negative controls, internal process controls (IPC; Life technologies) and external process control (salmonid elongation factor-1 α ; (Bruno et al., 2007)). For the *in situ* hybridisation assays, sections were hybridised with a digoxigenin (DIG)-labelled oligonucleotide probe specific to *N. perurans* as previously described (Young et al., 2008; Downes et al., 2015). In total, five samples were selected and screened by real-time PCR, two from 1995 and three from 1997 (Table 17).

5.4 Results and Discussion

This study confirms the presence of *N. perurans* in the first recorded outbreaks of AGD in Ireland (Rodger & McArdle, 1996; Palmer et al., 1997). The histopathology showed hyperplasia and fusion of the secondary lamellae as well as the formation of vesicles and the presence of amoeba (Fig. 17). In the corresponding sections the *N. perurans* probe hybridised with all trophozoites observed, further confirming the presence of this species of amoeba on the gills (Fig. 18). When tested for the presence of *N. perurans* each sample provided positive results with cycle threshold (Ct) values varying between 30.2 and 34.9. The Ct values for the external process control did not vary with average Ct values of 27.9 +/- 0.5 indicating that the DNA was successfully isolated consistently. Furthermore, the average Ct value for the internal process control was 28.4 +/- 0.3, which demonstrated no inhibition of the PCR reactions. The detection of the amoeba in archived samples is consistent with Young et al., (2008), which demonstrated *N. perurans* on the gills of AGD-affected fish from a number of geographical areas, including Irish samples from 2004. These results indicate that the amoeba has been present in Ireland since at least 1995.

Regarding the other pathogens, all samples from 1995 and 1997 tested positive for both *D. lepeophtherii* and SGPV, illustrating the widespread nature of this parasite and virus respectively. Two samples from 1997 were positive for *Ca. B. cysticola* and just a single sample positive for *T. maritimum* from 1995 (Table 17). With such a small selection of archived histopathological material from two Irish cases of AGD, any observations from these cases and how they apply to overall gill

disease in Ireland would be speculative. However, the Ct values recorded for *D. lepeophtherii* and SGPV were comparable to recent results for these pathogens from screening samples of Atlantic salmon gills from 2013 and 2014 (Chapter 4), further demonstrating that they are commonly found in marine farmed Atlantic salmon.

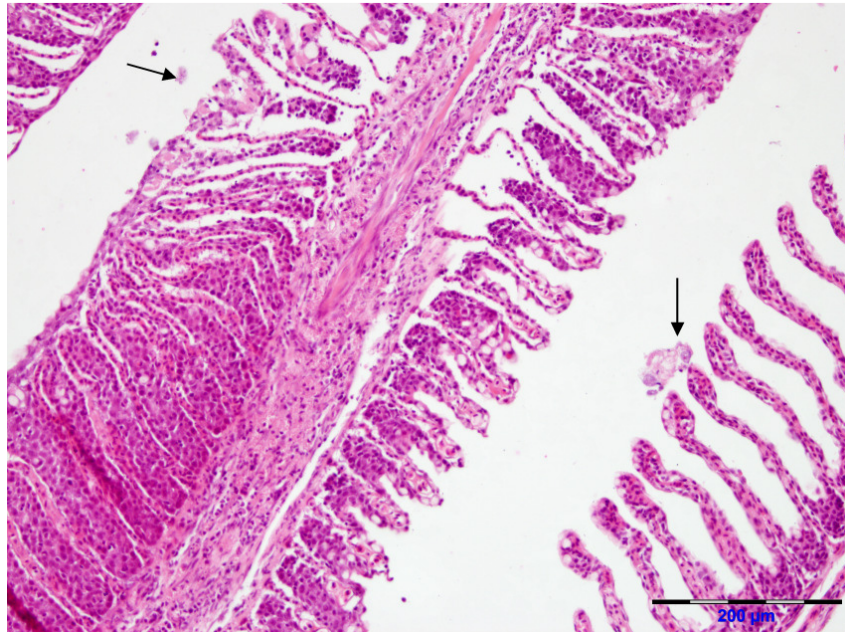


Figure 17. Gill section from Atlantic salmon sampled in 1995, showing pathology associated with AGD including hyperplasia and fusion and oedema. Numerous amoebae (arrows) are present (H&E).

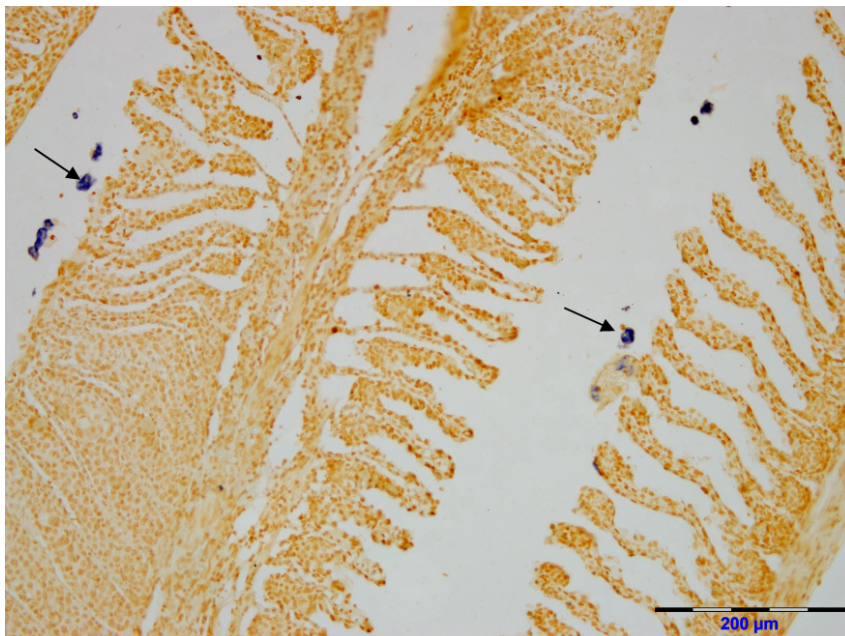


Figure 18. Atlantic salmon gill section from the same fish following *in situ* hybridisation using species-specific oligonucleotide probes for *Neoparamoeba perurans*. Reactive dark cells (arrows) indicate the presence of *N. perurans* trophozoites.

Table 17. Real-time PCR results (Ct values) for pathogens associated with gill disease from formalin-fixed paraffin-embedded Atlantic salmon gill samples collected during AGD outbreaks in 1995 and 1997 in Ireland.

| Sample | <i>N. perurans</i> | <i>Ca. B. cysticola</i> | <i>D. lepeophtherii</i> | <i>T. maritimum</i> | SGPV |
|--------|--------------------|-------------------------|-------------------------|---------------------|------|
| 1995 | 34.8 | Negative | 31.8 | Negative | 33.1 |
| 1995 | 33.3 | Negative | 29.9 | 35.5 | 37.1 |
| 1997 | 33.7 | 39.6 | 32.4 | Negative | 38.7 |
| 1997 | 31.9 | 35.3 | 31.5 | Negative | 37.7 |
| 1997 | 30.8 | Negative | 30.7 | Negative | 36.8 |

Initially, the conditions required for the development of AGD were believed to have been prolonged high sea temperatures and low rainfall as was observed in 1995 (Rodger & McArdle, 1996; Palmer et al., 1997). Currently, the peak phase in Ireland for the majority of new AGD outbreaks is June, July and August when sea temperatures are increasing. Indeed the majority of newly reported AGD outbreaks were preceded by abnormally high sea temperature (Oldham et al., 2016). However, amoebae have been observed on the gills of Atlantic salmon in Ireland throughout the year, often in the absence of clinical signs (Downes et al., 2015). Elsewhere, mortalities due to AGD have also been recorded at temperatures below 10°C (Douglas-Helders et al., 2001) and as low as 7°C (Steinum et al., 2008). *N. perurans* seemingly has the potential to be infectious over a wide range of temperatures but perhaps a more important risk factor in relation to AGD has been highlighted, which is the thermal tolerance of the host animal (Oldham et al., 2016).

The identification of *N. perurans* in cases of AGD from 1995 and 1997 raises the question regarding what has changed since 2011 to cause sustained outbreaks not previously observed in Ireland. Several gaps in knowledge in relation to AGD have been identified including relationships between amoebae concentrations, environmental parameters and bacterial load (Oldham et al., 2016). The identification of *N. perurans* as the aetiological agent of AGD has enabled the development and validation of sensitive real-time assays (Downes et al., 2017), which were not previously available. It is only now with these improvements in diagnostic technology that we are beginning to understand the true complexity of gill disease.

Gill disease is not a new issue (Rodger 2007). It is clear from this short study that the putative pathogens associated with gill disease today have been present in Atlantic salmon aquaculture in Ireland for over 20 years. The pathogens included in this study have also been found to be present in the gills without clearly causing pathology or affecting mortality (Chapter 4.). Koch's postulates have been proven for the involvement of *N. perurans* in AGD (Crosbie et al., 2012) and horizontal transmission has been shown to play a role in the development of gill disease (Wiik-Nielsen et al. 2017). However, the lack of viable cultures in axenic media or cell culture for gill disease-associated pathogens has inhibited the full investigation of the aetiology of gill disease and this should become a primary area of research in the coming years.

5.5 Author contributions

Conceived and designed the experiments: Jamie Downes, Evelyn Collins and Neil Ruane. Performed the experiments and sample analysis: Jamie Downes, Teresa Morrissey, Cathy Hickey and Evelyn Collins. Analysis of original samples: Roy Palmer and Margaret Rutledge. Writing and editing manuscript: Jamie Downes, Teresa Morrissey, Eugene MacCarthy, Ian O'Connor, Hamish Rodger and NR.

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Chapter 6

Discussion

6.1 Gill disease

Gill disease has consistently been a significant challenge for global marine salmonid aquaculture for over 30 years (Rodger & McArdle 1996, Palmer et al. 1997, Clark & Nowak 1999, Steinum et al. 2008, Mitchell & Rodger 2011, Ruane et al. 2013). In Ireland, over the period of 2003-2005 mortality due specifically to gill pathologies was estimated to be 12% (Rodger 2007). Since 2011/2012 the re-emergence of amoebic gill disease (AGD) caused by *Neoparamoeba perurans* is perhaps the most serious with respect to economic impact (Rodger 2014). The cause of gill disease is often multifactorial and there are numerous other putative pathogens which have been identified as having the potential to cause pathology (Mitchell & Rodger 2011). Concurrent infections of AGD, epitheliocystis and proliferative gill inflammation (PGI) are not uncommon (Gunnarsson et al. 2017). This research was initiated in order to provide the sector with the tools and information required to help inform management decisions and improve gill health.

6.2 Real-time PCR assay and longitudinal study

From the outset of this study, molecular diagnostics was identified as a key area of research. A number of real-time PCR methods were previously available for the detection of *N. perurans*, however, the capacity to detect the pathogen's DNA in low level (gill score) or subclinical infections had not been established. Furthermore, anomalies between traditional diagnostic techniques (gill score, wet prep and histology) and PCR based methods had been observed in the field. Issues also occurred in relation to false negative results (defined as a negative PCR result from a fish sample with clinical AGD) in a number of field samples tested by our laboratory (unpublished data). The need to develop and validate a reliable real-time TaqMan® PCR assay to detect *N. perurans* in Atlantic salmon gills was identified. The assay developed (Chapter 2) proved to be specific for *N. perurans* and showed no cross reactivity with any related species *N. pemaquidensis*, *N. branchiphila* or *N. aesturina*. It was highly sensitive and shown to be able to detect 2.68 copies of *N. perurans* DNA μl^{-1} . To further assess the performance of the real-time PCR assay and the practical implementation of molecular screening, it was utilised during a longitudinal study (Chapter 2). This study was carried out over 18 months of marine

production to investigate AGD in Ireland, with the aim of providing important information relating to the impact of the disease, potential risk factors and aetiology.

The longitudinal study demonstrated a clear infection pattern. AGD began to proliferate from 12 weeks post sea water transfer. Mortality due to AGD peaked in week 17 of the marine production which also coincided with the temperature rising above 15°C. The temperature range observed in this study was similar to that which occurred during the first outbreaks of AGD in Ireland (Rodger & McArdle 1996, Palmer et al. 1997), however, the role of elevated sea temperature as a risk factor for AGD has previously been questioned (Rodger 2014). Outbreaks of AGD have been recorded at temperatures as low as 7°C in Norway (Steinum et al. 2008), while in Ireland ongoing screening has recorded new outbreaks occurring in January (10°C) in S0 smolts. AGD has been recorded over a wide variety of temperatures and what may be more of a risk factor is the thermal tolerance of the host (Oldham et al. 2016). Temperature is perhaps particularly important during treatments as this imposes a handling effect resulting in acute stress on the fish with implications for water quality and fish welfare (Powell et al. 2015). Elevated temperatures also restrict the choice of treatments as hydrogen peroxide is not safe to use above 13.5°C (Adams et al. 2012, Rodger 2014).

The PCR assay also proved useful for monitoring amoebae levels during the post-treatment period of production. Gill tissue samples collected 4 days post-treatment confirmed a clear reduction in the percentage of PCR-positive fish (from 100% down to 10%). This is in line with previous observations of the reduction of the number of amoebae following freshwater bathing (Clark et al. 2003). The PCR assay was shown to be particularly beneficial in the role of monitoring the proliferation of the amoebae post-treatment. Gross and histological gill scoring can be misleading due to the presence of scarring from the previous infection and/or lack of experience. Following the first treatment an increase in PCR positive fish was observed within 3 weeks, however, it was 6 weeks post-treatment that an increase in the average gill and histology score was recorded. With some amoebae remaining on the gills and all cages having not received treatments simultaneously, re-infection can occur relatively quickly (Powell & Clark 2003, Adams & Nowak 2004). It has recently been reported that *N. perurans* has the ability to develop pseudocysts which could also play a role in the survival of amoebae when dislodged from the gills following treatment (Lima et al. 2016).

The results obtained during this study demonstrated that the PCR assay can be used as a tool for monitoring the progress of the disease in particular with the detection of the amoeba 3 weeks prior to detection via gross pathology. However, it must be noted that although the parasite was detected by PCR screening throughout the 18 month study period, mortalities associated with clinical AGD were only recorded in the first 12 months of the marine phase of production. Therefore, it is advised that the results from real-time PCR screening should be analysed in the overall health context of the site. Subsequently, gill screening conducted in Ireland for AGD using the technique developed in this study has identified cage to cage variation in the percentage of fish positive for *N. perurans* (Appendix I). While site to site variations in disease outbreaks are well noted (Soares et al. 2013), local effects on cage to cage variation must also be considered in relation to future studies and management practices. The cage environment has previously been identified as playing a role in gill disease where heavily fouled pens and pens with lower water exchange experienced more cases of clinically significant AGD (Rodger 2014). There is clearly the potential for real-time PCR to play a beneficial role as a tool to complement existing techniques for the monitoring of AGD. This study was somewhat limited by the number of fish that could be sampled, due to the destructive nature of the sampling. This was addressed in the subsequent study which validated a non-lethal sampling method (gill swabs) to determine its potential to further enhance monitoring capabilities of the aquaculture industry for AGD.

6.3 Non-destructive methodology

Since 2011 the industry in Ireland was quick to adopt the gross gill scoring method (Taylor et al. 2009) as a means for determining the severity of AGD on farms. Regular gross gill scoring is particularly suitable on Atlantic salmon farms that are consistently challenged by the disease, allowing operators to become familiar with gross presentation of lesions. Interpretation of the gill score can vary with experience of operators in addition to the presentation of non-AGD pathologies such as proliferative gill disease or gill necrosis. Gill scoring has also proved difficult and is less applicable for other fish species affected by AGD such as lumpfish (*Cyclopterus lumpus*), which are used as cleaner fish in Atlantic salmon cages (Haugland et al. 2016).

A number of sampling methods and molecular diagnostic assays were already being utilised in addition to the development of the assay in this study. There was a clear need to evaluate the suitability of the available molecular assays in conjunction with the most appropriate non-destructive methodology. Collaborating with CSIRO, Australia, this study aimed to assess two non-destructive methodologies (gill swab & gill biopsy) and compare the real-time PCR assays currently in use for the detection of *N. perurans*. The accurate and rapid detection of pathogens is essential for the implementation of an effective health management plan in aquaculture.

This study showed a clear improvement in sensitivity of molecular diagnostics when sampling with gill swabs in comparison to gill filament biopsies. In conjunction with the gill swabs, Protocol 1 in this study (Downes et al. 2017) (Chapter 3) performed most favourably across the majority of the attributes assessed and was deemed the most suitable assay. The applicability of this methodology was further demonstrated during an infection trial. *N. perurans* was detected by PCR prior to the development of gross symptoms and histology score. This verified the ability of the gill swab and PCR to detect sub-clinical cases. The development of an early detection method which is economical, sensitive and accurate enough to diagnose the disease in the early stages is very beneficial. Molecular diagnostics have been regularly employed to monitor the development of diseases in salmon aquaculture such as salmon alpha virus (Graham et al. 2006), infectious pancreatic necrosis virus (Soliman et al. 2009), in addition to numerous putative gill pathogens (Gunnarsson et al 2017).

This method (Downes et al. 2017) has also been deployed as part of a larger screening program for AGD in Ireland which demonstrates its applicability. Gill swabs are readily taken during gill screening with results rapidly produced once processed. The infection patterns observed during this screening of S1 smolts confirmed the results recorded in Chapter 1 (Appendix II). The majority of new outbreaks in Ireland are recorded between May to August. Likewise, in the majority of sites containing S0 smolts this period is also when new outbreaks occur. One site was identified as an exception to this with a new outbreak detected in January. The probable cause of this outbreak was most likely the proximity (~4km) of a different year class which was positive for AGD.

This diagnostic method (Chapter 3) has also shown potential as a means to evaluate treatment efficacy (F/W & H₂O₂). Gill screening conducted using this method has been utilised has shown that salmon treated for AGD with hydrogen peroxide (H₂O₂) observed little or no reduction in % positive for *N. perurans* and required retreatment two weeks later (Appendix III). Following a freshwater bath, gill swabs were negative by PCR for the presence of amoebae and a period of seven weeks elapsed before treatment was required again. These are incidental findings observed during the screening program, which highlights the potential of the molecular assay combined with non-destructive sampling to assess the efficacy of treatments for AGD. Furthermore, this method has been employed in the detection of a number of other putative gill pathogens such as *Desmozoon lepeophtherii* and *Candidatus Branchiomonas cysticola* which increases the scope and utility of this screening method (*Pers. Comm. Fish Vet Group*).

6.4 Multifactorial Gill Disease

Although the main focus of this research has been diagnostics in relation to AGD, numerous other pathogens associated with gill disease have been identified (Mitchell & Rodger 2011). Recently, there has been increased interest in the multifactorial aspects of gill disease as there is a deficiency of knowledge in relation to the occurrence and development of co-infections with many of these putative pathogens (Matthews et al. 2013, Oldham et al. 2016, Gunnarsson et al. 2017, Gjessing et al. 2017). In light of this, DNA previously extracted during the longitudinal study (Chapter 2) was assessed using real-time PCR assays for *Desmozoon lepeophtherii*, *Candidatus Branchiomonas cysticola*, *Tenacibaculum maritimum* and Salmon Gill Pox Virus (SGPV) in-order to investigate their occurrences during an AGD outbreak and determine whether there is any interaction between these putative pathogens.

All putative pathogens included in the study were detected at varying levels over the course of the 18 months of marine production (Chapter 4). *D. lepeophtherii* and *Ca. B. cysticola* were the most consistent pathogens detected throughout the study, as there was little variation in the percentage of fish positive for these pathogens following week 12. These putative pathogens have been detected in high prevalence in a number of other studies (Steinum et al. 2010, Mitchell et al. 2013,

Gunnarsson et al. 2017). *Tenacibaculum maritimum* was found to be significantly correlated with temperature ($r=0.48$, $p<0.05$) showing distinct seasonality as it increased with high temperatures and decreased over the winter months. Lower temperatures ($<15^{\circ}\text{C}$) are not optimal for the growth of *T. maritimum* (Smage et al. 2016), which may explain the seasonality observed in this study. Salmon Gill Pox Virus (SGPV) was highly sporadic throughout this study and was detected in the first sampling point, suggesting a carryover from freshwater stage of production. Horizontal transmission was recently demonstrated for SGPV under controlled conditions; however, SGPV transmission has been shown to be less effective than that of *Ca. B. cysticola*, which may explain the sporadic detections of the virus (Wiik-Nielsen et al. 2017). The modeling data from this study (Chapter 4) suggests that only *N. perurans* had a significant effect on gill pathology. None of the other putative pathogens appeared to have an effect on gill pathology. The modelling data also suggested that there was no significant interaction between the putative pathogens.

During the study, access was given to an archive of formalin fixed paraffin embedded (FFPE) histology blocks containing fixed gill tissue from the first Irish cases of AGD in 1995 and 1997. *In situ*-hybridisation and real-time PCR carried out on DNA from these blocks confirmed that *N. perurans* was the causative agent of the earliest AGD outbreaks in Ireland (Chapter 5). These blocks were also screened using real-time PCR assays for the putative pathogens identified in Chapter 4. This screening detected each of the putative pathogens associated with gill disease and verified that they have been present in Irish aquaculture for over 20 years. As capabilities in molecular diagnostics and other techniques increase, it has become easier to detect micro-pathogens. These studies are an evaluation of a small selection of samples from individual cases of gill disease in Ireland, thus observations made from these and how they might apply to the wider gill disease picture is speculative.

6.5 Future Research

Gill disease has been a persistent problem for the Atlantic salmon industry and it appears that amoebic gill disease has become endemic in Irish industry. This research developed a sensitive real-time assay for the detection of *N. perurans*, which was successfully employed to monitoring AGD throughout a production cycle

(Downes et al. 2015). There are a number of knowledge gaps for which the diagnostic method developed in this study (Downes et al. 2017) could be employed to help overcome. These include the relationship between *N. perurans* and environmental variables, distribution of the amoebae in the farm environment, net fouling, sediment and other potential reservoirs (Oldham et al. 2016). Furthermore, the molecular assay could be employed to investigate and compare site specific epidemiology including factors such as cage to cage prevalence, treatment (F/W & H₂O₂) efficacy, stocking density and the prevalence of *N. perurans* in cleaner fish. Such research would not just advance the knowledge and understanding of AGD but also aid in practical management decisions for fish health and welfare.

It has been shown in this study that agents associated with gill disease have been present in the Irish aquaculture industry for over 20 years. While great strides have been made in identifying agents of gill disease, some fundamental information is unknown and that is what constitutes a healthy gill biota. Such information could be determined by targeting wild salmon in order to compare organisms that naturally occur in the gills. The lack of viable cultures in axenic media or cell culture for gill disease-associated pathogens has also inhibited the investigation of the aetiology of gill disease and should continue to be a primary area of research in the coming years. As technology advances, the capabilities to detect pathogens have become more efficient. The advent of Next Generation Sequencing could potentially be a means of building on the data already collected in relation to the numerous putative gill pathogens and co-infections (Frey & Bishop-Lilly 2015).

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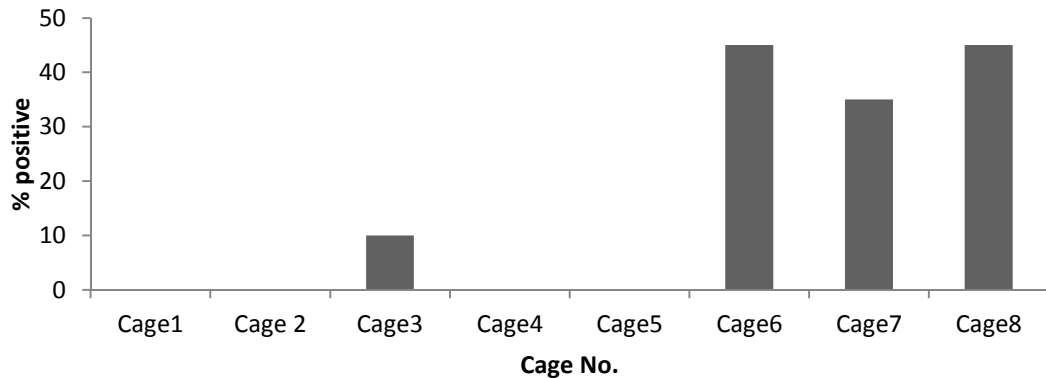
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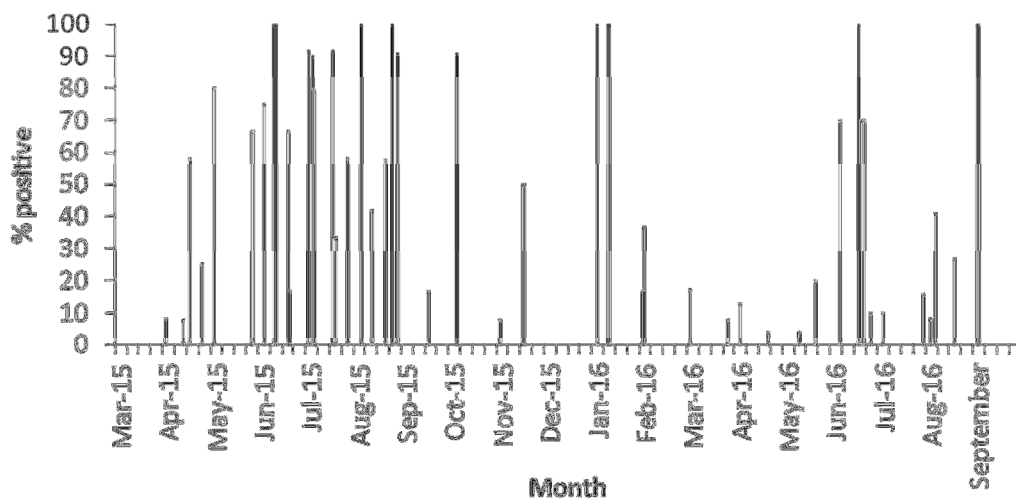
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Appendix I



Gill screening using the non-destructive gill swabs in conjunction with the PCR assay developed in this study has been conducted by the Marine Institute on a number of sites around Ireland. This gill screening has subsequently identified cage to cage variation in the levels of *N. perurans* detected in an individual site during an AGD outbreak. This is an important parameter to consider in the health management for AGD.

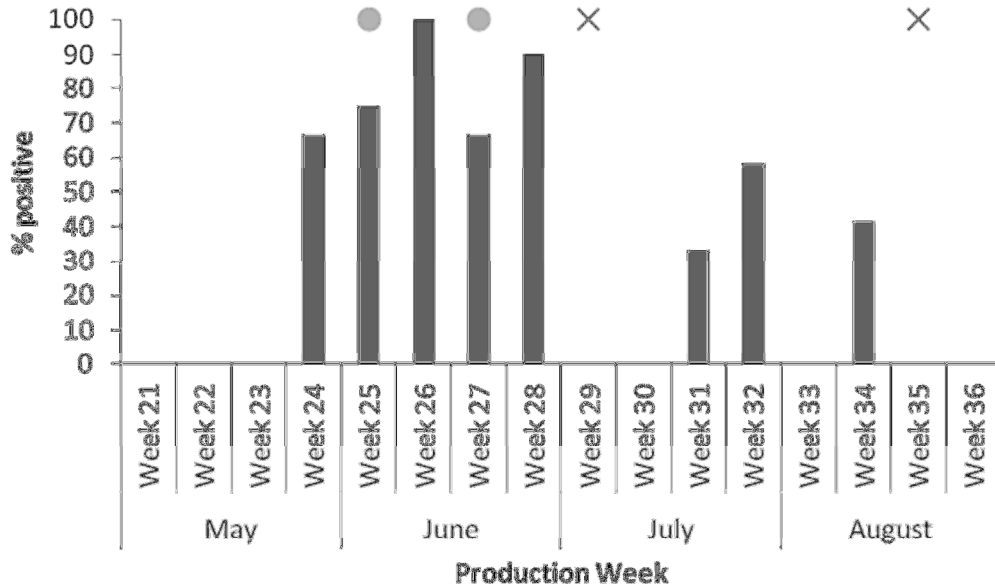
Appendix II



Gill screening using the non-destructive gill swabs in conjunction with the PCR assay developed in this study has been conducted by the Marine Institute on a number of sites around Ireland. This graph illustrates the percentage of gill swabs positive for *N. perurans* during screening for AGD in 7 sites containing Atlantic salmon stock in Ireland between 2015 and 2016. The screening has identified the main period for new cases of AGD is between May and September. However, a new

outbreak of AGD in January was recorded in one site containing S0 smolts. This outbreak was most likely due to the proximity of this site to another site containing a different year class.

Appendix III



The non-destructive PCR assay developed in this study has been used as a mean to evaluate the efficacy of treatments on sites. This graph illustrates the percentage of fish positive for the presence of *N. perurans* in S1 smolts over a treatment period. Treatments are indicated; (●) H₂O₂ 1200ppm for 20-30mins (X) Freshwater bath for 2-4 hours