Paramoeba perurans and AGD in Norwegian aquaculture: effect of freshwater treatment against AGD on gill health in commercial production of Atlantic salmon (*Salmo salar* L.) and experimental testing of virulence of *Paramoeba perurans*

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Abstract

Gill diseases are a major and increasing challenge with respect to fish health and welfare during the marine stage in Atlantic salmon farming. The causative agents for gill diseases are complex, and several pathogens are often present at the same time. Amoebic gill disease (AGD), caused by *Paramoeba perurans*, is strongly associated with gill diseases. To control and prevent development of the disease and mortality, freshwater treatment has shown to be the most effective method. Treatments and handling of the fish causes stressful situations affecting the fish negatively. How these stressful situations within the production affects the gills health and the associated microbiota is less known.

The main amin of this study was to map how stress in relation to commercial production (freshwater treatment) affects the gill health of Atlantic salmon. The gills were studied, and pathogens (prevalence and density) were mapped before and after treatment in addition to fish that died during treatment. Histopathological changes compatible with AGD lesions were observed, but there were no significant changes between the groups. The prevalence and density of some pathogens were significantly reduced after treatment, including *P. perurans*, *Ichthyobodo* spp, *Cand.* S. salmonis and *Cand.* P. salmonis. Other pathogens, including PRV1, *P. theridion*, SGPV, PMCV and *Cand.* B. cysticola, increased or had no change of prevalence and density in the gill tissue. The mortality prior to and during the treatment were high, but significantly reduced for the treated group. These results suggests that gill health prior to treatment, and presence of viruses will affect the outcome of the treatment and the mortality rate.

A complementary challenge experiment was conducted to test the possible effect of microbiota on the virulence of clonal cultures of *Paramoeba perurans* during an experimental challenge. Characterization of microbiota present in the culture media of *P. perurans* showed a large variation of bacteria species, dominated by *Vibrio splendidus* variants. Although *P. perurans* cultured in pure culture of *V. splendidus* grew as well as the amoeba in the original culture media, the clones where not able to establish infection on the gills. The results indicates that the clone's loss of ability to induce AGD in salmon could be related to the change in microbiota in the amoebas culture media or microbiota present on the salmon gill during infection with amoeba.

Abbreviations

AGD	Amoebic gill disease
ASPV	Atlantic salmon paramyxovirus
Cand. B. c.	*Candidatus* Branchiomonas cysticola
Cand. C. s.	*Candidatus* Clavichlamydia salmonicola
Cand. P. s.	*Candidatus* Piscichlamydia salmonis
Cand. S. s.	*Candidatus* Syngnamydia salmonis
CDG	Complex gill disease
CMS	Cardiomyopathy syndrome
Costia	Assay for *Ichthyobodo* spp.
Ct-value	Cycle threshold value
Dpi	Days post infection
Dpt	Days post treatment
E	Efficacy
EF1A	Assay for Atlantic salmon elongation factor
Epit	Assay for *Candidatus* Branchiomonas cysticola
FDRG	Fish Disease Research Group
FHF	Fiskeri- og havbruksnæringens forskningsfinansiering (Norwegian Seafood Research Fund)
F-primer	Forward primer
GD	Gill disease
H_2O_2	Hydrogen peroxide
HE	Hematoxylin and eosin
HSMI	Heart and skeletal muscle inflammation
ILAB	The Industrial and Aquatic Laboratory, Thormøhlensgate 55, 5008 Bergen, Norway
ISAV	Infectious salmon anemia virus
IPNV	Infectious pancreatic necrosis virus
L	Liter
mL	Milliliter
MNE	Mean normalized expression
MYA	Malt Yeats media

MYB	Malt yeast broth
N	Number of individuals
NE	Normalized expression
NTC	Non-template control
Nuc	Assay for *Paranucleospora theridion*
O_2	Oxygen
Parvi	Assay for *Parvicapsula pseudobranchicola*
PCh	Assay for *Candidatus* Piscichlamydia salmonis
PER	Assay for X cell parasite
PGI	Proliferative gill inflammation
PKX	Assay for *Tetracapsuloides bryosalmonae*
PMCV	Piscine myocarditis virus
Pperu	Assay for *Paramoeba perurans*
PRV1	*Piscine orthoreovirus* 1
Real time RT-PCR	Real time reverse transcriptase polymerase chain reaction
R-primer	Reversed primer
SAV	Salmonid Alphavirus
SGPV	Salmon gill poxvirus
sp.	Species (unkown) within the genera
spp.	Species within the genera
TB-tuf	Assay for Tenacibaculum spp.
UoB	Univeristy of Bergen
UoS	University of Sterling
µl	Microliter

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1 Introduction

1.1 Norwegian aquaculture

Since the start of aquaculture in Norway in the 1970s, the growth of the industry has expanded rapidly. According to Norwegian Directorate of Fisheries (2020), 1.36 million tons of Atlantic Salmon (*Salmo salar*) and 83 500 tons farmed trout (Rainbow trout and trout) were produced in Norway in 2019 with a value around 68.0 and 3.5 billion kroner, respectively. This is an increase of 6.4 % tons Atlantic salmon and 22.2 % tons farmed trout compared to 2018. Today, this makes Norway one of the leading countries when it comes to producing salmonids like Atlantic salmon and Rainbow trout. Therefore, the aquaculture industry is particularly important for Norway. The rapid growth of the industry causes challenges regarding welfare and fish health, and increased infection pressure. These challenges can inflict large financial losses in the industry due to expensive treatments, reduced growth and quality, poor fish health and welfare, and mortality. Infection with salmon louse (*Lepeophtheirus salmonis*, Krøyer, 1837), the viral diseases cardiomyopathy syndrome (CMS, Piscine myocarditis virus), pancreas disease (PD, Salmonid alphavirus), heart and skeletal muscle inflammation (HSMI, Piscine orthoreovirus), ulcerative bacterial diseases caused by *Moritella viscosa*, *Tenacibaculum* spp., or *Pasteurella* sp., and gill disease (multifactorial/complex) was some of the top ten challenges during the marine stage in 2020 in Norway (Sommerset, Bang Jensen, Bornø, Haukaas, & Brun, 2021). In order to expand the industry and production in Norway, these challenges must be solved.

1.2 Gill diseases

Gill diseases (GD) are a major and increasing challenge with respect to fish health and welfare in Atlantic salmon farming causing significant losses during production (Nylund *et al.*, 2011; Bloecher *et al.*, 2018; Downes *et al.*, 2018; Herrero, Thompson, Ashby, Rodger, & Dagleish, 2018). The etiology for GD is often multifactorial and complex, and it is difficult to identify a primary pathogen causing the disease (Nylund *et al.*, 2011; Bloecher *et al.*, 2018; Downes *et al.*, 2018; Herrero *et al.*, 2018). Due to the gills position and physical structure, the constant and intimate contact with surrounding environment, they are particularly susceptible to infection and physical damage (Bloecher *et al.*, 2018; Herrero *et al.*, 2018). Fish gills have several essential physiological and critical functions including respiration, osmoregulation, pH regulation and ammonia secretion (Steinum *et al.*, 2010; English *et al.*, 2019). Fish with

compromised function of gills may display signs of poor respiratory function, loss of appetite, poor food conversion and welfare, and increased mortality rates (Herrero *et al.*, 2018). GD is not a notifiable disease, which makes it difficult to map the extent of the disease in the industry. According to Sommerset *et al.* (2021), multifactorial/complex gill disease is a severe problem in the industry during the marine phase.

Several factors, such as environmental conditions (temperature, water quality, oxygen), handling procedures (transport, treatments, sorting), the general health of the fish and presence of pathogens, may play a key role in the inception of gill disease (Nylund *et al.*, 2011; Herrero *et al.*, 2018). Numerous agents, both infectious and non-infectious, have been associated with gill disease including viruses, parasites, bacteria, phyto- and zooplankton species and biofouling organisms at the cage nets (Nylund *et al.*, 2011; Herrero *et al.*, 2018). Establishing the cause of multipathogen and multifactorial gill disease is difficult. Today general terms for gill disease are used inconsistent, including epitheliocystis, proliferative gill inflammation (PGI), proliferative gill disease (PGD) and complex gill disease (CGD) (Herrero *et al.*, 2018). Epitheliocystis is characterized by large cyst-like epidermal lesions with intracellular bacteria at the lamellae, and is primarily associated with Chlamydia-like organism, such as *Candidatus* Piscichlamydia salmonis, *Candidatus* Clavichlamydia salmonicola, *Candidatus* Syngnamydia salmonis, and the β-proteobacteria *Candidatus* Branchiomonas cysticola (Hoffman, Dunbar, Wolf, & Zwillenberg, 1969; Nylund, Kvenseth, & Isdal, 1998; Draghi *et al.*, 2004; Karlsen *et al.*, 2008; Steinum *et al.*, 2010; Mitchell *et al.*, 2013; Nylund *et al.*, 2015). PGI is used as a term to describe outbreaks of gill disease in smolts along the southwest coast of Norway in the autumn, often associated with the microsporidian parasite *Desmozoon lepeophtherii* (syn. *Paranucleospora theridion*) (Hoffman *et al.*, 1969; Nylund *et al.*, 1998; Draghi *et al.*, 2004; Karlsen *et al.*, 2008; Steinum *et al.*, 2010; Mitchell *et al.*, 2013; Nylund *et al.*, 2015). PGI is also characterized by a combination of histopathological changes like inflammation, necrosis, hyperplasia of epithelial cells and lamellar vascular changes (Kvellestad, Falk, Nygaard, Flesja, & Holm, 2005). Proliferative gill disease (PGD) is a non-specific term referring to proliferative changes of lamellar epithelial cells in the gills when it comes to histology (Nylund *et al.*, 2008; Herrero *et al.*, 2018). Herrero *et al.* (2018) and Boerlage *et al.* (2020) stated that PGD is mostly diagnosed on gross lesions in the field. The term "complex gill disease" (CGD) is associated with non-specific clinical gill diseases occurring in marine farmed Atlantic salmon from the end of summer to early winter (Herrero *et al.*, 2018). CGD is referred to when multifactorial etiology and variable histopathology is observed (Herrero *et al.*, 2018). In earlier published

articles, terms like PGI and PGD is encompassed by CGD (Herrero *et al.*, 2018; Boerlage *et al.*, 2020).

In addition to the gill diseases above, other pathogens are associated with gill diseases in Norway, often identified at the same time. Salmon gill poxvirus (SGPV) is a virus often found in the gills when the fish show signs of gill disease (Herrero *et al.*, 2018; Boerlage *et al.*, 2020). Atlantic salmon paramyxovirus (ASPV) has been associated with gill disease and PGI but is now less consistently related with the disease (Munday, Foster, Roubal, & Lester, 1990; Nowak, 2012). Parasites, such as *Paramoeba perurans* and *Ichthyobodo* spp. are associated with gill disease and believed to cause Amoebic gill disease (AGD) and Ichthyobodosis, respectively (Young, Crosbie, Adams, Nowak, & Morrison, 2007; Isaksen, Karlsbakk, Watanabe, & Nylund, 2011). Other bacteria associated with gill disease is *Tenacibaculum maritimum*, causing problems within farming in Canada, and has lately been detected in Norwegian farms (Frisch *et al.*, 2018).

1.3 Amoebic gill disease

Since the first description of amoebic gill disease (AGD) in marine farmed Atlantic Salmon (*Salmo salar* L.) and Rainbow trout (*Oncorhynchus mykiss*) in the mid 1980s, the disease has developed to be one of the most serious challenge of salmonid farming in Australia (Tasmania) (Kube, Taylor, & Elliott, 2012; Nowak, 2012; Nowak, Valdenegro-Vega, Crosbie, & Bridle, 2014; Hvas, Karlsbakk, Mæhle, Wright, & Oppedal, 2017). AGD is a disease that have been reported worldwide in farmed Atlantic Salmon and Rainbow trout, and is causing significance concern in Northern Europe (Wiik-Nielsen *et al.*, 2016). Today, the disease is a serious problem which causing economic losses as a result of reduced growth, mortality, and treatment expenses, but also reduced quality of the carcasses and feed conversion (Munday *et al.*, 1990).

AGD is a disease affecting the gills and compromises its crucial physiological functionality. Gill changes as a result of the colonization of the amoeba, including severe mucosal epithelial hyperplasia, hypertrophy, interlamellar vesicle formation, lamellar fusion and necroses (Kube *et al.*, 2012; Nowak, 2012; Nowak *et al.*, 2014; Hvas *et al.*, 2017). The colonization on the gill surfaces is visible to the naked eye as multifocal white mucoid patches (Munday *et al.*, 1990). The patches are a good indicator for controlling the disease development and to preliminary diagnosis of the infection (Bridle, Crosbie, Cadoret, & Nowak, 2010; Kube *et al.*, 2012; Rodger,

2014; Bridle, Davenport, Crosbie, Polinski, & Nowak, 2015; Oldham, Rodger, & Nowak, 2016). Clinical signs of AGD are anorexia, lethargy, more rapid ventilation rate and loss of appetite (Young *et al.*, 2007; Young, Dyková, Snekvik, Nowak, & Morrison, 2008; Mitchell & Rodger, 2011; Oldham *et al.*, 2016; Hvas *et al.*, 2017; Kim *et al.*, 2017). If the disease is untreated, increased susceptibility to other diseases and associated mortality during treatments and handling will eventually occur (Kent, Sawyer, & Hedrick, 1988; Munday, Zilberg, & Findlay, 2001; Dyková *et al.*, 2005). As stated by Nowak *et al.* (2014), when mucus containing *P. perurans* is sloughed into the water column, the amoeba can be transmitted horizontally and colonize on nearby fish gills.

1.3.1 *Paramoeba perurans*

Paramoeba perurans (syn. *Neoparamoeba perurans*) is a marine, free-living, amphizoic, facultative and opportunistic ectoparasite shown to be the causative and etiological agent of AGD (Young *et al.*, 2007; Karlsbakk *et al.*, 2013; Rodger, 2014). The amoeba is classified and taxonomic located within the Genus *Paramoeba*, Family Paramoebidae, Order Dactylopodida, Subclass Flabellinia, Class Discosea and Phylum Amobeozoa (Kudryavtsev, Pawlowski, & Hausmann, 2011; Smirnov, Chao, Nassonova, & Cavalier-Smith, 2011). In literature, *Neoparamoeba perurans* is often used synonymous with *P. perurans*. According to Kudryavtsev *et al.* (2011); Feehan, Johnson-MacKinnon, Scheibling, Lauzon-Guay, and Simpson (2013), it is likely impossible to divide *Paramoeba* and *Neoparamoeba* into two monophyletic genera due to no well-defined distinction within morphology and genetics between then genera.

The free-living and round amoeba often measures 20-30 µm in diameter, while the adherent form of the amoeba has been measured to 41-56 µm (Young *et al.*, 2007; Karlsbakk *et al.*, 2013; Rodger, 2014). The amoeba lacks clear structure on the cell surface, such as hexagonal glycostyles or surface scale, but the plasma membrane is well defined (Young *et al.*, 2007; Oldham *et al.*, 2016; Wiik-Nielsen *et al.*, 2016). The amoeba-cell contains two types of cytoplasm; transparent ectoplasm which can form extended pseudopods from the cell (Page, 1987) and granulated endoplasm which includes organelles, nutrition vacuoles and cell nucleus (3.3 – 6.0 µm) (Young *et al.*, 2007; Wiik-Nielsen *et al.*, 2016). The morphology of *P. perurans* may vary between round cells and polymorphic cells with clear pseudopods (Wiik-Nielsen *et al.*, 2016). Additionally, the amoeba has one or more intracellular perinuclear bodies

(parasomes) close to the nucleus (Young *et al.*, 2014). The parasomes are eukaryotic endosymbionts closely related to parasitic flagellates (*Ichthyobodo*), known as *Perkinsiella* amoebae-like organisms (PLOs) (Page, 1987; Dyková, Fiala, Lom, & Lukeš, 2003; Young *et al.*, 2007; Young *et al.*, 2014; Wiik-Nielsen *et al.*, 2016). The function of the parasome is unknown, however it is believed that the symbiosis is obligate and mutualistic (Dyková *et al.*, 2003; Young *et al.*, 2014). Young *et al.* (2014) suggest that the parasome is of importance for inflicting disease. In a study performed by Nylund *et al.* (2018b), the intracellular bacteria *Ca.* Syngnamydia salmonis (*Chlamydiales, Simkaniaceae*) was found in clones of *P. perurans*, indicating the bacterium could multiply in the amoeba.

The complete and natural lifecycle of *P. perurans* is still unknown (Oldham *et al.*, 2016). The amoebae are not able to form double-walled true cysts, but they can form rounded cyst-like cells called pseudocysts (Wiik-Nielsen *et al.*, 2016). Pseudocysts are believed used for protection in situation with limited area for adhesion or available nutrition during osmotic stress (Dyková, Figueras, & Peric, 2000; Wiik-Nielsen *et al.*, 2016; Lima, Taylor, & Cook, 2017).

Different amoeba species have been associated with and suggested as etiological agents of AGD in the past, such as *Paramoeba pemaquidensis* and *Paramoeba branchiphila* which have been isolated form fish with AGD (Kent *et al.*, 1988; Munday *et al.*, 2001; Dyková *et al.*, 2005). In 2006, salmon at a production site in Sogn and Fjordane suffered high mortalities associated with gill disease and a new species of *Paramoeba* was detected (Young *et al.*, 2008; Feehan *et al.*, 2013; Oldham *et al.*, 2016). A description of this new *Paramoeba*, *P. perurans*, was given by (Kudryavtsev *et al.*, 2011; Smirnov *et al.*, 2011) based on detection of the same amoeba in Tasmanian salmon farming. Young *et al.* (2008) found that *P. perurans* was the only amoeba that was detected within gill lesions from four different species of fish in five different countries. A challenge conducted in 2010 by Crosbie, Bridle, Cadoret, and Nowak (2012) resulted in successfully infection of naive Atlantic salmon with cultured *P. perurans*, and fulfilled Koch's postulate.

Several factors can affect the virulence of the amoeba. One factor suggested to be of significance with respect to virulence is the composition of the microbiota (Benedicenti, Secombes, & Collins, 2019b). It is believed that extracellular products (ECP) produced by the amoeba also affects the virulence and is important for inducing AGD (Bridle *et al.*, 2015). According to a study conducted by Kindt (2017), the virulence of the amoeba increases when

the temperature increases from 12 to 16 °C. Additionally, passages of the clones held in cultures over a long period of time have shown to reduce the virulence of the clones (Jellett & Scheibling, 1988; Visvesvara, Moura, & Schuster, 2007; Veríssimo, Maschio, Correa, Brandelli, & Rott, 2013; Bridle *et al.*, 2015)

1.3.2 Pathology

The pathology of AGD is primarily limited to the gills, where clinical signs can be observed as loss of appetite, altered swimming behavior, lethargy, extruded operculum and increased respiratory rate due to respiratory distress (Kent *et al.*, 1988; Munday *et al.*, 1990; Steinum *et al.*, 2008; Boerlage *et al.*, 2020). The amoeba can adhere to healthy epithelium in the gills, and further proliferate and colonize at the site (Zilberg & Munday, 2000; Adams & Nowak, 2004a). AGD is characterized clinically by local changes on the gill surfaces as white, mucoid patches (hyperplastic epithelial lesions) (Munday *et al.*, 1990). This is a result of the host response to an infection by *P. perurans* due to irritation and increased excretion of mucus (Munday *et al.*, 1990; Clark & Nowak, 1999; Adams & Nowak, 2001; Marcos-López & Rodger, 2020). These patches are used as a tool to assess the development and severity of the disease in Atlantic salmon indicating the level of damage on the gills (Taylor, Muller, Cook, Kube, & Elliott, 2009b). This method is also known as scoring of the gills.

Histopathological studies describe changes in the gill tissue, where AGD is associated with hyperplasia of the epithelial cells, fusion and vacuolization of the secondar lamellae, and formation of interlamellar vesicles or lacunae (cavitation), hyperplasia of mucus cells and some infiltration of inflammation cells (Kent *et al.*, 1988; Munday *et al.*, 1990; Adams & Nowak, 2001, 2003; Adams & Nowak, 2004a; Steinum *et al.*, 2008; Mitchell & Rodger, 2011; Crosbie *et al.*, 2012). The amoeba can be observed in the interlamellar lacunae, or near the epithelial cells in the affected areas (Adams & Nowak, 2001; Leef, Harris, & Powell, 2005; Steinum *et al.*, 2008; Bustos *et al.*, 2011; Mitchell & Rodger, 2011). Studies have shown that the pseudopodia of *P. perurans* may penetrate the lamellar epithelium, a mechanism to improve the attachment to the hosts tissue or increase the conditions for the amoeba in contact with the host (Lovy *et al.*, 2007; Wiik-Nielsen *et al.*, 2016). Reduction of chloride cells or chloride cells sloughed off gill tissue can be observed in affected area with lesions (Adams & Nowak, 2003). Multifocal necrosis in the liver have been registered in severe cases of infection (Rodger, 2014).

Pathology associated with AGD will reduce the functional surface of the gills, which will make it hard for the fish to perform respiration and osmoregulation, followed by respiratory acidosis (Powell, Fisk, & Nowak, 2000; Fisk, Powell, & Nowak, 2002). A study by Powell *et al.* (2000) indicated that the mortality is not caused by the respiratory failure as Atlantic salmon with AGD in hypoxic environment can compensate the reduced gill surface with other physiological mechanism such as increasing the gill perfusion or redistribution of the blood flow within the gills. Study of the cardiovascular system have shown an association between AGD and vascular hypertension as well as morphological changes and reduced cardiac output (Powell, Nowak, & Adams, 2002; Leef, Harris, Hill, & Powell, 2005). Therefore, it is believed that cardiovascular stress and vasoconstriction due to increased vascular resistance and chronic hypertension is of importance for mortality associated with AGD (Leef, Hill, Harris, & Powell, 2007; Powell, Reynolds, & Kristensen, 2015). These circulatory disturbances can correlate to the pathology observed in the liver, especially in the late stages of the infection (Wiik, 2020). It is also believed that mortality associated with AGD occurs when the fish is treated or when exposed to handling and stress (Powell *et al.*, 2015).

Although it is known that the fish have an immune evasion strategy when infected by *P. perurans*, there is a lack of knowledge for all the immune responses (Benedicenti, Collins, Wang, McCarthy, & Secombes, 2015). It is shown that *P. perurans* elicits classical inflammatory responses in the gills, and expression of cellular markers, such as antigen presenting cells (B- and T-cells), increases in the early stages of infection (Pennacchi, Leef, Crosbie, Nowak, & Bridle, 2014). Elevated proinflammatory cytokines in the later stages of infection is observed (Bridle, Morrison, Cupit Cunningham, & Nowak, 2006; Morrison, Young, & Nowak, 2012). Additionally in the late stages of infection, Benedicenti *et al.* (2015) suggest an immune evasion strategy similar to an infection of helminthic parasites to suppress cell-mediated killing mechanisms.

1.3.3 Risk factors

P. perurans has no host specificity and can infect several species all over the world, therefore it is difficult to find clear trends that trigger outbreak of AGD globally (Young *et al.*, 2007; Mitchell & Rodger, 2011; Oldham *et al.*, 2016; Hvas *et al.*, 2017). Studies have shown that high temperature (>12 °C) and salinity are two important environmental factors affecting the outbreak of disease in Atlantic salmon (Clark & Nowak, 1999; Douglas-Helders, Saksida, Raverty, & Nowak, 2001). Outbreaks in Tasmania have been associated with high temperatures

between 15-20 °C during the summer and autumn (Munday *et al.*, 1990). According to Oldham *et al.* (2016), many AGD-cases have indicated that there is an correlation between periods of elevated temperature and establishment of AGD in new areas. This correlates to the first case of AGD in Norway in 2006, where the sea temperature was registered 3.5 °C higher than normal (Steinum *et al.*, 2008). At higher sea temperature, Atlantic salmon is more susceptible to pathogens and diseases due to different physiological challenges that follows the critical temperature limit rather than that the virulence of the amoeba has changed (Clark & Nowak, 1999; Oldham *et al.*, 2016). There are indications that the amoeba has adapted to colder temperature. Although most outbreaks are registered at >12 °C, outbreaks of the disease have been registered at temperatures near 6-7 °C (Clark & Nowak, 1999; Douglas-Helders *et al.*, 2001; Steinum *et al.*, 2008; Rodger, 2014; Mo, Hytterød, Olsen, & Hansen, 2015).

Salinity is considered the most important environmental factor associated with outbreaks of AGD, where salinity >25 ‰ is associated with risk of outbreaks and salinity >32 ‰ has a greater risk of inducing outbreaks of AGD (Clark & Nowak, 1999; Douglas-Helders *et al.*, 2001; Munday *et al.*, 2001; Steinum *et al.*, 2008). *P. perurans* has a low tolerance for freshwater (Oldham *et al.*, 2016). Bridle *et al.* (2015) assessed the ability of the amoeba to attach in cell culture of CHSE-214 cells in terms of salinity, where a significant higher number of amoebae were attached in 30 ‰ compared to 20 ‰. The optimal temperature and salinity for growth of *P. perurans* under experimental conditions is at 15 °C and 35 ‰, and the thresholds for growth is within the temperature- and salinity ranges of 4-8 °C and 20-25 ‰ (Collins, Hall, Fordyce, & White, 2019).

It is believed that other factors, such as size, density and immune status of the fish, genetics, treatments conducted, presence of pathogens on the gills and water quality, may be of importance for the development of disease (Clark & Nowak, 1999; Adams & Nowak, 2001; Munday *et al.*, 2001; Bermingham & Mulcahy, 2004; Douglas-Helders, Weir, O'Brien, Carson, & Nowak, 2004; Bermingham & Mulcahy, 2007; Rodger, 2007; Taylor, Wynne, Kube, & Elliott, 2007; Crosbie, Bridle, Leef, & Nowak, 2010; Kube *et al.*, 2012). Challenge experiments with *P. perurans* conducted at UoB and ILAB indicated that there is variation between populations of salmon, as well as within the different populations (Kindt, 2017). Douglas-Helders, Nowak, Zilberg, and Carson (2000), have shown that *Paramoeba* spp. can survive on the gills of dead salmon for >30 hours, as well as proliferate and infect naive fish, therefore dead salmon needs to be removed due to risk of spreading the amoeba in the cages (Oldham *et*

al., 2016). The severity of the disease will be affected by the dosage of amoeba, where increased concentration of *Paramoeba* sp. is correlated with increased number of lesions on the gills (Zilberg & Munday, 2000). Although the effect on the disease is not fully understood, it is believed that the fish have different susceptibility for the amoeba due to different genetical resistance mechanisms (Taylor, Kube, Muller, & Elliott, 2009a; Kube *et al.*, 2012).

1.3.4 Diagnostics and detection

The gill scoring system developed by Taylor *et al.* (2009b) is a system used to estimate the severity of AGD in the gills of Atlantic salmon, and to indicate clinical diagnosis by studying the lesions (patches) on the gills. The system uses a scale from 0 to 5, where 0 equals to no sign of infection and 5 is extensive lesions. Studies have shown that the method is not directly transferable to other marine species, including ballan wrasse and lumpfish (Dahle, 2015; Haugland, Olsen, Rønneseth, & Andersen, 2017; Dahle *et al.*, 2020a).

Lesions and white patches on the gills are the hosts response to pathogen on the gills, and may be caused by other pathogens, as well as *P. perurans* (Adams, Ellard, & Nowak, 2004; Nylund *et al.*, 2011; Quaglio *et al.*, 2016). Therefor the scoring system cannot be used alone to confirm AGD on the fish as the only diagnostic method. The method most used for identifying the presence of the amoeba is by real-time RT-PCR of gill tissue with specific assay for *P. perurans* (Fringuelli, Gordon, Rodger, Welsh, & Graham, 2012; Downes *et al.*, 2015; Nylund *et al.*, 2018b). Histological examination of the gills can confirm the presence of amoeba-like organisms associated with hyperplastic lamellar fusion and formation of interlamellar vesicles or lacunae, as well as hyperplasia of mucus cells (Adams & Nowak, 2001; Munday *et al.*, 2001; Adams *et al.*, 2004; Fringuelli *et al.*, 2012; Rodger, 2014). Other methods used to identify the presence of amoeba is by wet preparation from the affected gills, studied in microscope.

1.4 Host range, Transmission and Reservoirs

It seems that the host range of *P. perurans* is not specific (Oldham *et al.*, 2016; Hvas *et al.*, 2017), and the amoeba infects farmed Atlantic salmon (*Salmo salar*), Rainbow trout (*Oncorhyncus mykiss*), Coho salmon (*Oncorhynchus kisutch*), turbot (*Scophthalmus maximus*), ayu (*Plecoglossus altivelis*), ballan wrasse (*Labrus bergylta*), lumpfish (*Cyclopterus lumpus*), and black seabream (*Acanthopagrus schlegelii*) (Munday *et al.*, 1990; Steinum *et al.*, 2008; Young *et al.*, 2008; Karlsbakk *et al.*, 2013; Nowak & Archibald, 2018; Steigen *et al.*, 2018).

Due to low host specificity and globally widespread presence of the amoeba, it is suggested that the amoeba is opportunistic (Young *et al.*, 2008; Oldham *et al*., 2016). Although many hosts are susceptible for the amoeba, it does not necessarily result in AGD as some species may be more resistant to infection of *P. perurans,* which is observed within ballan wrasse (Karlsbakk *et al*., 2013).

Although the mechanisms for transmission of the amoeba between different localities are unclear (Nowak, Bryan, & Jones, 2010), horizontal transmission is identified in challenge experiments by direct exposure of amoebae on naïve fish or the usage of cohabitants (Crosbie *et al.*, 2012; Dahle, 2015; Haugland *et al.*, 2017). Bridle *et al.* (2015) found that the salmon could act as a reservoir for the amoeba and possible transfer infection with currents to other localities.

The presence of *Paramoeba* spp. have been identified in marine and estuarine sediments, on the nets in cages, and in some marine organisms such as crabs and sea urchins (Tan, Nowak, & Hodson, 2002; Crosbie, Nowak, & Carson, 2003; Dyková *et al*., 2005; Dyková *et al.*, 2007; Nowak & Archibald, 2018). Even though naked amoebae (Lobosa) are present in the marine environment from open ocean to estuary along the coasts, the natural reservoir of *P. perurans* is still unknown (Page, 1987; Rogerson & Laybourn-Parry, 1992; Oldham *et al.*, 2016). Previous studies have shown that amoeba originated from the gills of ballan wrasse can infect salmon (Dahle *et al.*, 2020a). Karlsbakk *et al.* (2013); Haugland *et al.* (2017); Dahle *et al.* (2020a) found cleaner fish without patches to be carriers of the amoeba, which can act as potential reservoir in cages together with Atlantic salmon (Oldham *et al.*, 2016; Steigen *et al.,* 2018). In addition, *P. perurans* have been identified from the surfaces of salmon lice *(Lepeophtheirus salmonis*), however it is unclear whether this is a significance as reservoir or important for transmission (Nowak *et al.*, 2010).

1.5 Treatments

If elevated gill scores compatible with AGD are identified on a locality, it is important to treat the fish in early stages of the disease to control it and avoid high mortality, as well as achieve the best possible effect. AGD may reduce welfare of the fish and cause mortality if left untreated (Nowak *et al.*, 2014), and studies by Rodger (2014) have shown the more severe the outbreak, the more difficult it will be to achieve an effective treatment. The most common methods used

for treating AGD is by freshwater- or hydrogen peroxide bath. Freshwater bathing has shown to be most efficient and been used as a treatment against AGD since the disease was first described in Tasmania in mid-1980s (Powell *et al.*, 2015; Oldham *et al.*, 2016). The treatment is performed by transferring the fish into a well boat with freshwater (salinity <3 ‰), alternatively in net pens with a closed tarpaulin, for a duration of 2-3 hours (Parsons, Nowak, Fisk, & Powell, 2001; Rodger, 2007; Adams, Crosbie, & Nowak, 2012; Rodger, 2014; Powell *et al.*, 2015). Handling and crowding of fish during the treatment can be stressful for the fish, where respiratory distress, hypoxia and mortality may follow (Parsons *et al.*, 2001; Powell, Parsons, & Nowak, 2001). Freshwater bathing is used both therapeutic and prophylactic (Rodger, 2014; Powell *et al.*, 2015). The need of nearby freshwater sources and logistics associated with transport of the water can be demanding in terms of costs and quality of treatment (Munday *et al.*, 2001; Kube *et al.*, 2012; Rodger, 2014; Powell *et al.*, 2015). Although the treatment shows significant effect on the gill lesions, it is normal to perform several treatments during the production cycle due to possible reinfections (Clark & Nowak, 1999; Clark, Powell, & Nowak, 2003; Adams & Nowak, 2004b). The possibility of a shorter treatment duration of Atlantic salmon sublethal for *P. perurans* as an alternative to lethal treatments were assessed by Wright *et al.* (2018). The results suggested that targeting detachment of amoeba rather than death of amoeba can be used as a method to control AGD. Freshwater bathing cannot be used on the marine cleaner fish present in the cages, therefore, to maintain good welfare in the cages, brackish water needs to be used to remove the amoeba. Dahle *et al.* (2020a) found treatment with brackish water (15 ‰) over several days to be effective.

Hydrogen peroxide (H_2O_2) have been used as an alternative to treat AGD affected fish, and studies have proven effect against the amoeba (Hytterød *et al.*, 2017a). In addition, hydrogen peroxide is used against salmon lice (Bruno & Raynard, 1994; Grave, Horsberg, Lunestad, & Litleskare, 2004). Adams *et al.* (2012) indicated in a study that hydrogen peroxide baths are equally efficacious as freshwater on mild cases of AGD, and that H_2O_2 will not leave any residue due to breaking down into oxygen and water. The treatment is performed in the same way as freshwater treatments, by well boats or closed tarpaulin. Due to smaller therapeutic window for hydrogen peroxide treatments, treatments performed at temperature >12 ºC may lead to bleeding in the gills and elevated mortality rate (Hytterød *et al.*, 2017a). Treatments performed at lower temperature in early stages of AGD can be effective in removing the amoeba (Hytterød *et al.*, 2017a). However, studies have shown that both freshwater and hydrogen peroxide bathing will not remove the amoeba completely in severe cases of AGD, and the

disease may progress after treatment (Parsons *et al.*, 2001; Hytterød *et al.*, 2017a; Martinsen, Thorisdottir, & Lillehammer, 2018).

1.6 Microbiota

Although there is paucity of knowledge, it is believed there is a connection of microbiota on the gills and the severity of AGD. The relationship between *Paramoeba* and bacteria is not fully understood, but it is suggested that the relation is more complex than the bacteria only being a source of nutrition (Nowak & Archibald, 2018). *P. perurans* is often associated with coinfections with pathogenic bacteria or microbial dysbiosis on the gills (Egan & Gardiner, 2016; Nowak & Archibald, 2018). Merrifield and Rodiles (2015) demonstrated that the gills of both freshwater and marine fish hold a wide range of bacteria genera. Microbiota have been suggested to affect the growth and virulence of different clones and cultures of *P. perurans* due to large variations of bacteria species and significant differences in the composition (Collins *et al.*, 2017; Benedicenti *et al.*, 2019b). Other factors affecting the microbiota on the gills can be stress factors, including water quality, temperature changes, crowding of fish, nutritional deficiencies, parasite infections or primary viral infections (Cahill, 1990). In addition, there is evidence supporting that season variation (Al-Harbi & Uddin, 2007) and poor water quality (Masouleh, Sharifpour, & Arani, 2006) affects the gill microbiota.

The relation of how microbiota on the gills affects fish health, infections of *P. perurans* and treatments is still not fully understood. It is possible that treatments could cause quick changes in the aquatic environment which can affect the microbiota present on the gills. During challenge experiment, the surrounding environment is controlled, hence the fish can allocate all energy needed to re-establish the gill microbiota. Pathogen present on the gills in commercial production will challenge the fish health and potential reduce the gill health. Microbial dysbiosis on the gills caused by treatments can possibly make the fish more susceptible for infection, and in worst cases lead to mortality.

1.7 Aim for study

The main aim of the study was to map the effect of freshwater treatment, against AGD, on the gill health of Atlantic salmon. The gill health and presence of pathogens (prevalence and density) were mapped before and after treatment. In addition, the prevalence and density of

pathogens on the gills of salmon dying during treatment were compared to the same parameters before and after treatment.

Hypothesis H_0: Freshwater treatment will not have a negative effect on the gill health.

The secondary aim of this study was to test the possible effect microbiota may have on the virulence of clonal cultures of *Paramoeba perurans* during experimental challenge.

Hypothesis H_0: The microbiota will not influence the virulence of *P. perurans*.

2 Materials and Methods

The material from this project is divided in two parts. Some of the material is part of an FHF-project (Norwegian Seafood Research Fund); "Effects of treatment against salmon lice, amoeba gill disease (AGD) and cleaning of nets on gill health for farmed salmon". The aim for the project is to study how stress during production, such as mechanical treatment against salmon lice, freshwater treatment against AGD and cleaning of nets, affects the gill health. In this master thesis the effect of freshwater treatment against AGD (*Paramoeba perurans*) in normal production of farmed salmon was studied. The other part of the material included in this study is from a challenge experiment aimed to map the importance of different microbiota, in cultures of *P. perurans*, on the development of AGD in Atlantic salmon (*Salmo salar*).

The material was analyzed at the Fish Disease Research Group (FDRG) laboratories, University of Bergen (UoB). Additional material collected during the field work were sent to other research groups (Pharmaq Analytiq and University of Sterling (UoS)) participating in the FHF-project. Results from their work are no presented in this master thesis.

2.1 Challenge: Study of the possible importance of bacteria, in culture media for clones of *P. perurans*, on the development of AGD.

The challenge was conducted at ILAB, The High Technology Centre in Bergen, from the 15th of October to the 17th of November 2020. The fish used in this challenge was Atlantic salmon (*Salmo salar*, L.) from Stofnfiskur Iceland. They were kept in the tanks (8 tanks containing 140 liter full sea water) for one week before challenge. At the start of the experiment, the average weight of the fish was 87 grams. 303 fish was used in the challenge.

Throughout the challenge, the water temperature was set to 16 °C and the light regime 12 hours light: 12 hours dark. The water flow in the tanks were set to 400 L/hours, and the oxygen saturation was set to >82 % during the challenge. Temperature and salinity in the tanks were controlled daily, as well as the behavior of the fish. To ensure good environmental conditions in the tanks, excessive feed and moribund fish were removed daily.

This challenge was approved by The Norwegian Food Safety Authority (application 24564).

2.1.1 Isolates (*P. perurans*) and bacteria (*Vibrio splendidus)*

The ST19/15Pp isolate of *P. perurans* was obtained in October 2015 from farmed Atlantic salmon at Hitra, Sør-Trøndelag (Kindt, 2017). During a challenge experiment in 2015 the isolate inflicted a moderate degree of pathology on the salmon (Røed, 2016). The clone is characterized as an amoeba of relative normal size where pseudocysts are rapidly formed in the floating stage and it is growing well in cultivation (Kindt, 2017). The H20/16Pp isolate of *P. perurans* was isolated in March 2016 from farmed Atlantic salmon in Hordaland (Kindt, 2017). The clone grows well in cultivation, and consist of many small cells, both adherent and floating stages (Kindt, 2017).

In this challenge, two clones of *P. perurans* was used, one high-virulent (H20/16Pp) and one low-virulent (ST19/15Pp) (Kindt, 2017). The two clones of *P. perurans* have been maintained in the original microbiota media since isolation and the challenge experiments performed during 2016. During the period from autumn 2016 until the start of this challenge experiment in October 2020 the clones had been through 96 (H20/16Pp) and 103 (ST19/15Pp) passages. A month before the start of the challenge both clones were divided in two, one line were kept in the original microbiota media while the other line were transferred to a medium containing a pure culture of *Vibrio splendidus*. The four clonal cultures went through six passages in the month before the challenge the 15th and 16th of October 2020. The culture media consisted of; malt-yeast-broth (MYB; 0.01 % malt extract, 0.01 % yeast extract, 34 PSU saltwater) and bacteria (the original microbiota or a pure culture of *V. splendidus*) incubated at a temperature of 16 ºC.

2.1.2 Challenge of salmon with clonal cultures of *P. perurans*

After one week in the tanks, the fish was challenged with *P. perurans* (table 2.1). The supply of water was stopped and the level of the water in the tanks was reduced to 100 L during the infection. The dose was set to be 1000 amoeba/L for each tank (a total of 100 000 amoeba/tank). CASY Model TT Cell counter (Innovatis, Roche Diagnostics) was used to calculate the correct amount of living amoeba and to prepare the inoculum (Haugland *et al.*, 2017). Before transferring the amoeba to the tanks, all the surfaces in the bottle were scraped with cell scraper so all of the amoebae could be transferred. The contents of the bottles were then directly distributed in the tanks and rinsed with the water from the tanks one time. The behavior of the fish was observed during the infection. The exposure tanks were oxygenated during the challenge. After one hour the water supply was turned on.

Table 2.1 Overview of each challenge group in each tank.

Tank	Date of challenge	Challenge material
Tank 1	16.10.2020	Clone H20/16Pp grown in pure culture of *V. splendidus*.
Tank 2	15.10.2020	Clone H20/16Pp grown in normal media (obtained from gills of farmed salmon during first isolation of *P. perurans* in 2016).
Tank 3	16.10.2020	Clone ST19/15Pp grown in pure culture of *V. splendidus*.
Tank 4	15.10.2020	Clone ST19/15Pp grown in normal media (obtained from the gills of farmed salmon during first isolation of *P. perurans* in 2015).
Tank 5	16.10.2020	Cloned culture of *V. splendidus* (the same isolate used for culturing the clonal cultures of *P. perurans*).
Tank 6	15.10.2020	Normal media containing microbiota obtained from the gills of farmed salmon during first isolation of H20/16Pp in 2016.
Tank 7	15.10.2020	Normal media containing microbiota obtained from the gills of farmed salmon during first isolation of ST19/15Pp in 2015.
Tank 8	16.10.2020	Control; exposed to bacteria- and amoeba-free malt Yeast media.

2.2 Freshwater treatment against AGD

A 10-hour freshwater treatment of Atlantic salmon (*Salmo salar*) suffering from AGD, associated with *P. perurans*, was carried out at a locality in Rogaland County in September 2020. The name and the location of the farm (LB) is anonymized (figure 2.1).

Figure 2.1 Map with approximate location of locality LB. Map obtained from Kommunekart.com.

Locality LB consists of four sea cages, but only two of the cages contained Atlantic salmon when the treatment took place. The fish had been transferred to sea at the end of October 2019. At the day of sampling, the number of fish at the locality was 290 000 – one cage with 143 224 fish, and the other with 146 776 fish. Prior to this AGD-treatment, there had been three delousing treatments; one with SkaMik (August 2020) and two with Thermolicer (July and June 2020). The fish at the locality was diagnosed with amoebic gill disease (AGD).

Only one of the sea cages received freshwater treatment and the other was left untreated. The mortality prior to the treatment was 100-300 fish each day for the last fourteen days. During treatment, the mortality in the treated cage was 1500 fish. A month after treatment, the mortality of the treated cage was 10-60 fish each day (25.09-04.10) and 150-200 fish each day (25.09-01.10) for the untreated cage.

The treatment was performed using a well boat Ronja Polaris. Average weight of the fish at the day of treatment was 2448 grams. Sea temperature at the day was 14.9 °C.

2.3 Sampling form the challenge- and the field experiment

During the challenge experiment a total of four samplings were carried out distributed over eight sampling-days. The fish from the challenge experiment was euthanized by percussive stunning.

Two visits to the farm with AGD was done before and after treatment with freshwater: 1) before treatment (22.09.2020), and 2) after treatment (30.09.2020). During the first visit tissue samples (gills, kidney, and heart) were collected from 30 newly euthanized salmon collected from the cage that were to be treated with freshwater one hour later, while during the second visit similar samples were taken from both treated cage (N = 30) and from untreated cage (N = 30). Fish that died during the freshwater treatment (N = 28) were collected and stored at -25 °C for later sampling in the FDRG laboratories, UoB. The fish sampled at locality LB was euthanized by an overdose of anesthetic.

2.3.1 Gill score

When monitoring the development of gill lesions during the challenge and in the field, a scoring system that estimate the severity and extent of AGD were used (Taylor *et al.*, 2009b). The

system uses a scale from 0 to 5, were 0 indicates no sign of infection and 5 indicates extensive lesions (table 2.2). At all samplings during the challenge, all the gill arches, including left and right, front and back, were studied thoroughly and scored individually according to table 2.2. At all samplings in field, all gill arches on left side were studied thoroughly and scored individually according to table 2.2. All equipment and glows were washed and changed between each tank and sampling.

Table 2.2 Gross gill score system used to assess the severity of AGD (Taylor *et al.*, 2009b).

Infection level	Gill score	Gross description
Clear	0	No sign of infection and healthy light color
Very light	1	1 white spot, light scarring and undefined necrotic streaking
Light	2	2-3 spots/small mucus patch
Moderate	3	Established thickened mucus patch or spot groupings up to 20% of gill area
Advanced	4	Established lesions covering up to 50% of gill area
Heavy	5	Extensive lesions covering most of the gill surface

2.3.2 Challenge

Sampling during the challenge was conducted at the FDRG laboratories, UoB. The number of fish taken at each sampling date is given in table 2.3. Weight (g) and length (cm) of the fish, external lesions or ulcers and gill score (table 2.2) was registered during each sampling. If any external lesions or ulcers were observed, samples were taken. Gill tissue the size of a “match-head” was taken from the apical part of the second gill arch on the left side for real time RT-PCR analysis. The rest of the second gill arch was taken as backup. The samples for analysis were transferred to 2.0 mL Safe-Lock Tubes (Eppendorf) and backups was transferred to 2.0 mL Nunc-tubes (Thermo Scientific) and stored on dry ice before transfer to -50 °C.

Table 2.3 Sampling dates during challenge period. Number of days post infection in parenthesis, N = number of fish sampled.

	1st sampling	**2nd sampling**	**3rd sampling**	**4th sampling**
Tank 1	04.11.20 (19 dpi) N = 5	10.11.20 (25 dpi) N = 2	12.11.20 (27 dpi) N = 10	17.11.20 (32 dpi) N = 22
Tank 2	03.11.20 (19 dpi) N = 5	09.11.20 (25 dpi) N = 2	11.11.20 (27 dpi) N = 10	16.11.20 (32 dpi) N = 19
Tank 3	04.11.20 (19 dpi) N = 5	10.11.20 (25 dpi) N = 2	12.11.20 (27 dpi) N = 10	17.11.20 (32 dpi) N = 20
Tank 4	03.11.20 (19 dpi) N = 5	09.11.20 (25 dpi) N = 2	11.11.20 (27 dpi) N = 10	16.11.20 (32 dpi) N = 20
Tank 5	04.11.20 (19 dpi) N = 5	10.11.20 (25 dpi) N = 2	12.11.20 (27 dpi) N = 10	17.11.20 (32 dpi) N = 22
Tank 6	03.11.20 (19 dpi) N = 5	09.11.20 (25 dpi) N = 2	11.11.20 (27 dpi) N = 10	16.11.20 (32 dpi) N = 21
Tank 7	03.11.20 (19 dpi) N = 5	09.11.20 (25 dpi) N = 2	11.11.20 (27 dpi) N = 10	16.11.20 (32 dpi) N = 22
Tank 8	04.11.20 (19 dpi) N = 5	10.11.20 (25 dpi) N = 2	12.11.20 (27 dpi) N = 10	17.11.20 (32 dpi) N = 21

2.3.3 Field

Sampling during the freshwater treatment was taken at site in the following groups: before treatment and after treatment (treated and untreated groups). The fish that died during treatment were collected and frozen (-25 °C) by those working at the site. 30 fish was sampled from each group, except for the fish that died during treatment (N = 28), in total 118 salmon.

When sampling, weight (g) and length (cm) of the fish, lice, any external lesions or ulcers and gill score (table 2.2) was registered for all groups except the ones that died during treatment. The second gill arch at the left side was transferred and fixed in neutral buffered 10% formalin (Sigma-Aldrich) for histology, and the third gill arch was sampled for immunology and sent to University of Sterling. Samples of gill- and kidney tissue were taken for RNA extraction and real-time RT-PCR by taking a sample size of a “match-head” respectively from the apical part of the second gill arch on the right side and the head kidney. The samples were transferred into 2.0 mL Safe-Lock Tubes (Eppendorf) on dry ice during the sampling and stored at -25 °C for further analysis at UoB. The rest of the second gill arch, in addition to samples of heart and kidney, was transferred to 2.0 mL Nunc-tubes (Thermo Scientific) as backups and stored at -50 °C at FDRG laboratories, UoB.

Table 2.3 Dates for sampling in connection with AGD treatment at site LB. Number of days post treatment (dpt) in parenthesis, N = number of fish sampled.

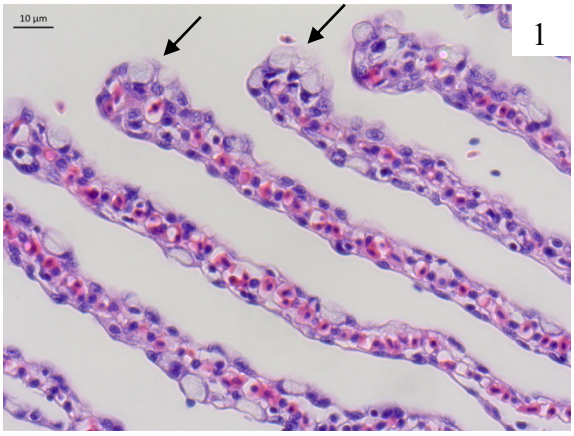
Before treatment	Dead during treatment	After treatment (treated)	After treatment (untreated)
22.09.20 N = 30	22.09.20 N = 28	30.09.20 (8 dpt) N = 30	30.09.20 (8 dpt) N = 30

2.4 Histology

Gills samples taken for histology were sent to Pharmaq Analytiq (Bergen) for preparation of the sections. The tissues were embedded in paraffin and by standard procedures prepared for microscopy (histological analysis) (Gamble & Bancroft, 2008). The gills were cut in the sagittal plane with a thickness of 2 µm and placed on poly-L-lysine coated slides (SuperfrostPlus, Thermo Scientific, Germany). The slides were stained with hematoxylin and eosine (HE) (Dahle *et al.*, 2020b).

The histological sections were studied to identify possible changes of cell- and tissue structures, and also to identify possible pathogens on the gills. All samples before and after the freshwater treatment was studied, and the histological sections were scored based on a simplified system of identifying changes in gills for this FHF-project (Kvåle, 2020). Five primary lamellae were scored at each section, and the scoring system addressed eleven normally occurring pathological changes in the gill tissue with a score range = 0-3 (figure 2.2). The different scores were given based on the percentage affected tissue that was studied. Score 0 indicates no changes in the tissue, score 1 is given with less than 10 % of changes (mild changes), score 2 indicates between 10-50 % changes (moderate changes), and score 3 is given if more than 50 % of the tissue is affected (extensive changes). As for hyperplasia of mucus cell, the score is given based on the average amount of mucus cells on each secondary lamella: 3 or less mucus cell indicates score 1, 5-8 mucus cells indicate score 2, and more than 8 mucus cells indicates score 3. The score for thickened distal filament is given based on the percentage affected primary lamellae, where score 1 indicates less than 10 % is affected, score 2 indicates 10-50 % is affected, and score 3 indicates that more than 50 % is affected.

The scoring of gills was done using Leica DM500 light microscope and Zeiss® Axio Scope.A1 with Axiocam 105 color-camera. The images were processed in ZEN lite 2012 v.1.1.2.0.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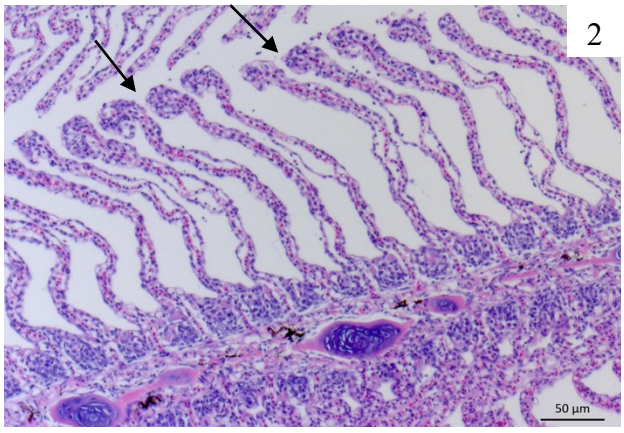
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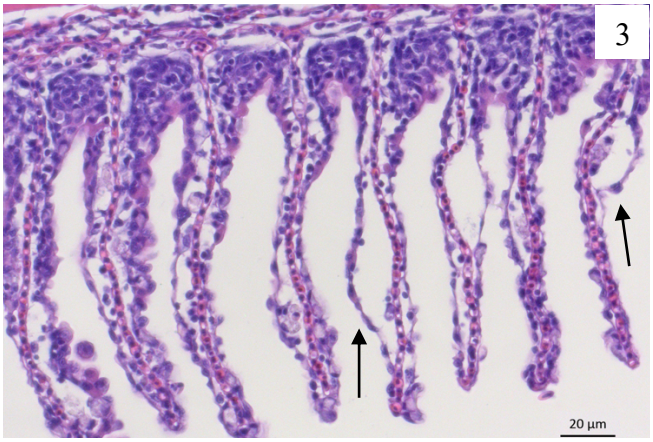
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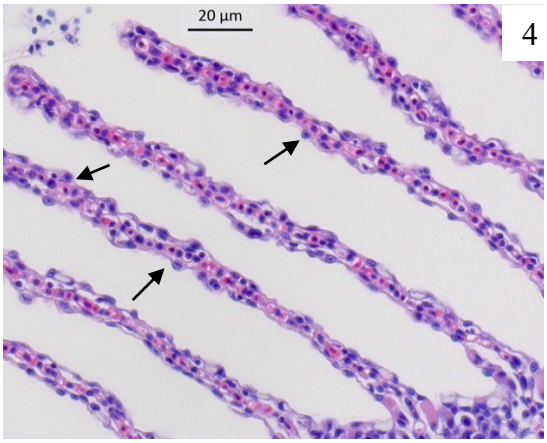
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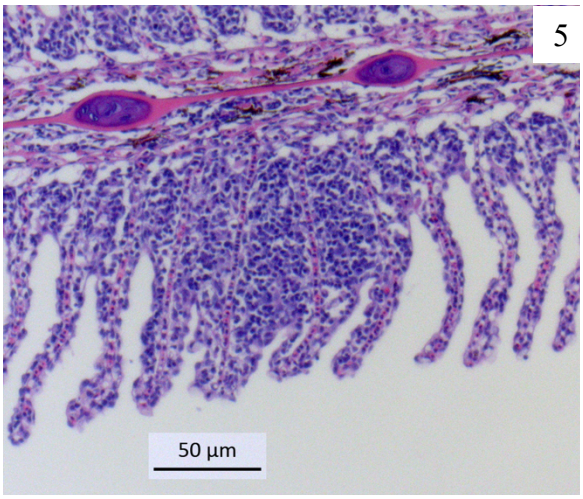
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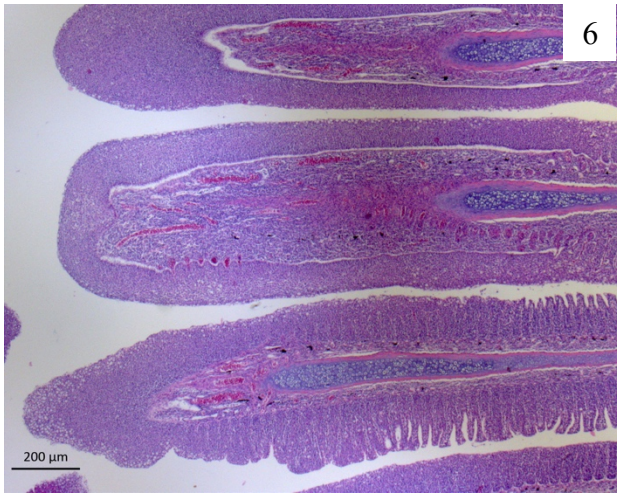
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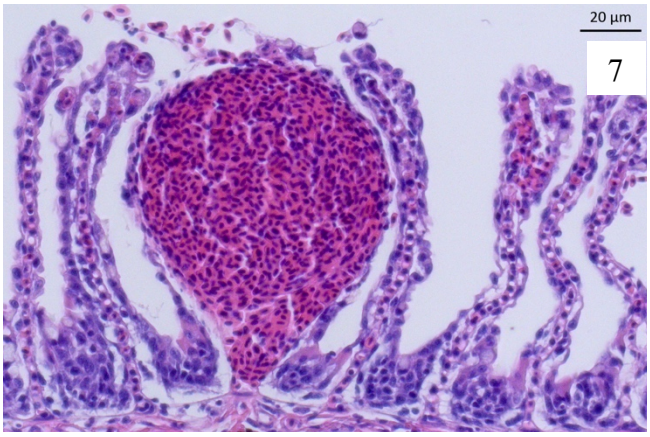
200 µm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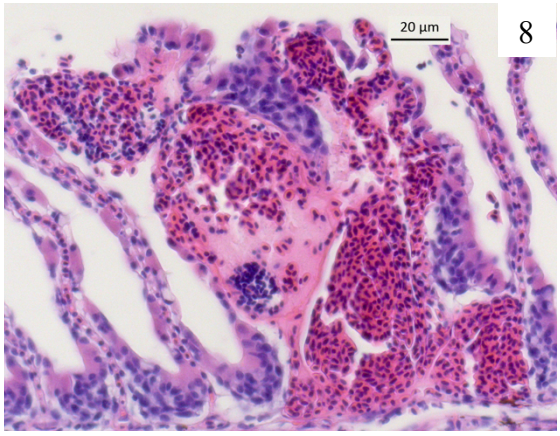
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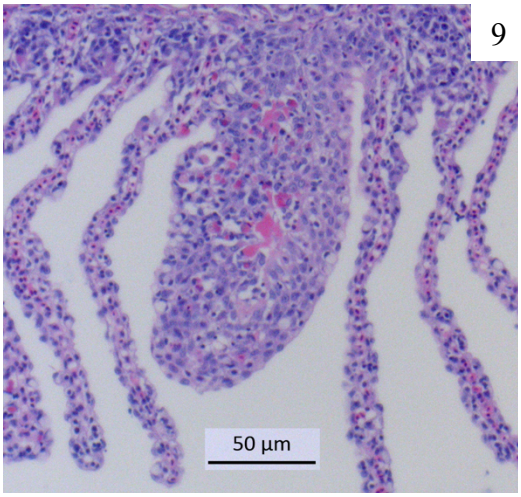

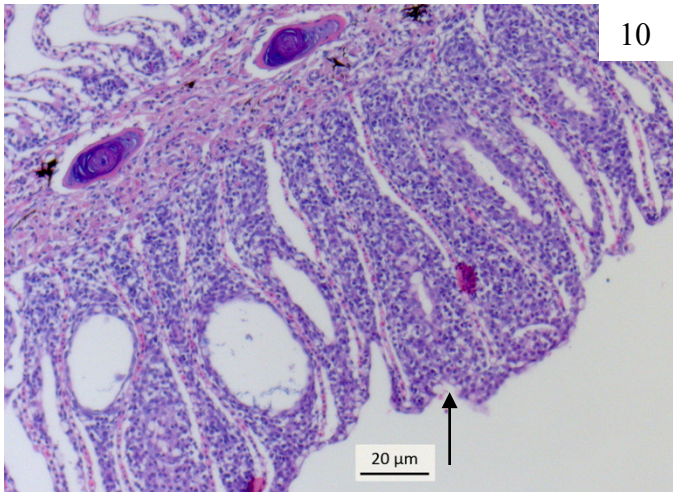

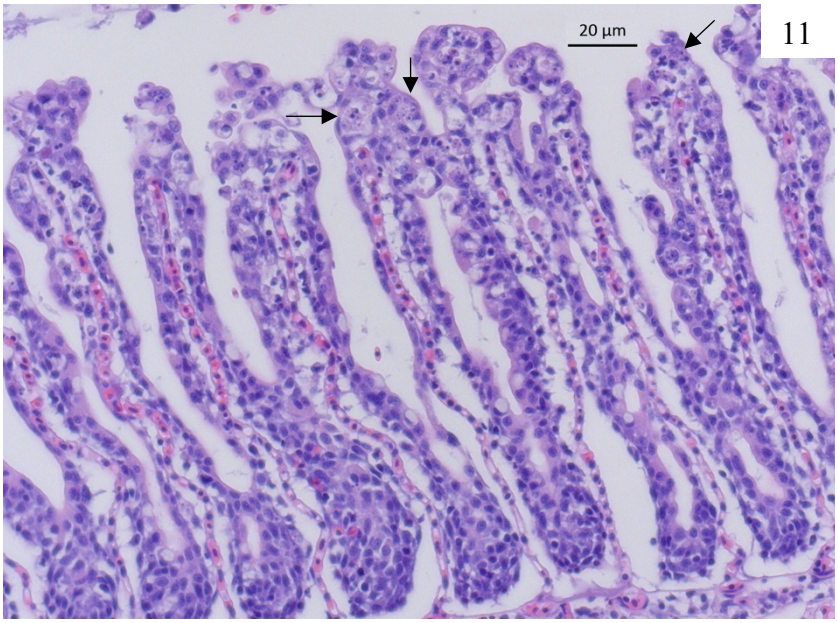

Figure 2.2 Eleven normal occurring pathological changes in the gill tissue illustrated histological. Arrow indicates changes. Scale is given in each figure. **1**: Mucus cell hyperplasia. **2**: Clubbing. **3**: Lifting. **4**: Epithelial cell hypertrophy. **5**: Epithelial cell hyperplasia. **6**: Thickening of distal primary lamellae. **7**: Fresh aneurism. **8**: Bleeding aneurism. **9**: Old aneurism. **10**: Inflammation. **11**. Necrosis.

2.5 Real time RT PCR analysis

2.5.1 RNA extraction

The RNA extractions were carried out as described by Gunnarsson *et al.* (2017a) with some modifications. TRIzol Reagent (Sigma) was used in most cases, but a few extractions were carried out using QIAzol® Lysis Reagent (Qiagen).

First, 1000 µL TRIzol or QIAzol was added to tubes (Eppendorf Safe Lock 2,0 mL) containing tissue samples before it was homogenized in TissueLyser II (Qiagen) for 3 minutes with 30 oscillation per second. The samples were incubated in room temperature for five minutes and spun down before adding 200 µL chloroform (Sigma-Aldrich) and then the tubes were vigorously shaken for 15-20 seconds. Further, the samples were incubated in room temperature for five more minutes, and then centrifuged (Thermo Scientific™ Heraeus Fresco™ 21) for 15

minutes at 12 000 x g and 4 °C. The samples separated into tree different phases: aqueous phase at the top, interphase and organic phase at the bottom. 450-500 µL of the aqueous phase, which contains the RNA, is pipetted over to Axygen Microtubes 1.5 mL tubes containing 500 µL isopropanol (Antibac) and mixed carefully. The tubes were then incubated for 10 minutes before centrifugation for 15 minutes at 12 000 x g and 4 °C to precipitate RNA-pellets from the solution. To increase the quality of the RNA extraction, the pellet was washed twice with ethanol (VWR). The supernatant was removed from the tube, 1.0 mL 75 % ethanol was added, and the pellet was washed on all sides by vortex for a few seconds before centrifugated for 5 minutes at 12 000 x g at 4 °C. Ethanol was removed, and the washing was repeated with 100 % ethanol. The pellet was then air dried for 10 minutes before dissolved in 100-150 µL RNase-free water (Sigma-Aldrich) pre-heated to 70 °C. While extracting RNA, one negative control (RK) followed the same steps as described, but without tissue in the tube. One RNA extraction control was added for each 10 tissues sample to control possible contaminations. The samples were then stored at -25 °C until further analysis.

As a part of controlling the quality and RNA-concentration (ng/µL) of the samples, the absorbance of a random selection of samples was measured in a spectrophotometer (NanoDrop™ 1000, Thermo Scientific).

2.5.2 Real-Time RT-PCR

Real-Time RT-PCR is an efficient method for detection of RNA from specific microparasites. The extracted RNA from the samples were analyzed by using AgPath-ID™ One-Step RT-PCR Kit (ThermoFisher Scientific) and Applied Biosystems® QuantStudio™ Real-Time PCR System (ThermoFisher Scientific). By combining mastermix of specific primers and probes, as well as the template in a reaction plate with 96 wells (Applied Biosystems® MicroAmp® Optical 96-Well Reaction Plate), the Real-Time RT-PCR will give an amplification curve that indicates how much of the template is present in the sample. First, 10.5 µL of the mastermix was added to the wells. The mastermix consist of 2X RT-PCR Buffer (Applied Biosystems®), F-primer, R-primer, probe (TaqMan), 25X RT-PCR Enzyme mix (Ambion) and RNase-free water (Sigma-Aldrich) (table 2.4). Further, 2.0 µL of the template is added to the wells. In addition to the templates, one negative control (RK) and one non-template control (NTC) was analyzed for each assay.

Table 2.4 The components of the mastermix with associated volume (µL) for the assays. The volume corresponds to one analyze. The final concentration for primer and probe is given in parenthesis.

Component	General	Pperu	POX MCP
2X RT-PCR Buffer	6.25	6.25	6.25
F Primer	1.00 (800 nM)	1.0 (800 nM)	0.5 (800 nM)
R Primer	1.00 (800 nM)	1.0 (800 nM)	0.5 (800 nM)
Probe	0.22 (175 nM)	0.28 (175 nM)	0.28 (175 nM)
25X RT-PCR Enzyme mix	0.25	0.25	0.25
Water (RNase free)	1.78	1.72	2.72
RNA Template	2	2	2
Total volume	12.5	12.5	12.5

The first step in the reactions is revers transcription at 45 °C for 10 minutes. Then the temperature rises to 95 °C for 10 minutes where deactivation of reverse transcription and activation of polymerase takes place. The last step, amplification of target template, runs for 45 cycles at 95 °C (DNA dissociation) for 15 seconds and 60 °C (annealing and elongation) for 45 seconds. The amplifications curves are given in the program QuantStudio Design™ & Analysis Software v1.5.1. The threshold line for all analysis was manually set to 0.1.

Gill- and kidney samples collected from the field experiment were tested against different assays (table 2.5) to detect which pathogens were present. The assay for elongation factor (EL1A, table 2.5) in salmon was used as a reference gene (Olsvik, Lie, Jordal, Nilsen, & Hordvik, 2005). All gill samples from the challenge experiment were tested using the assays EF1A, Pperu, Perk and SCh.

Table 2.5 Primers and probes for assays used in Real-time RT-PCR analysis. Efficacy is given in the references.

Assay	Primer	Sequence	Reference
Salmonid alphavirus (nsP1)	Probe	CTG GCC ACC ACT TCG A	Hodneland and Endresen (2006)
	Forward	CCG GCC CTG AAC CAG TT	
	Revers	GTA GCC AAG TGG GAG AAA GCT	
Infectious salmon anemia virus (Segment 7)	Probe	CAC ATG ACC CCT CGT C	Plarre, Devold, Snow, and Nylund (2005)
	Forward	TGG GAT CAT GTG TTT CCT GCT A	
	Revers	GAA AAT CCA TGT TCT CAG ATG CAA	
Piscine orthoreovirus 1 (PRV1-M2)	Probe	CTG GCT CAA CTC TC	Nylund *et al.* (2018a)
	Forward	CAA TCG CAA GGT CTG ATG CA	
	Revers	GGG TTC TGT GCT GGA GAT GAG	
Piscine orthoreovirus 3 (PRV3-S1)	Probe	CGA AGY ATA ATG AAG A	A. Nylund, pers. com.
	Forward	GCG ACG CCT TAG AGA CAA CA	
	Revers	CRA GAT CAC CAG TGG TCT TT	
Piscine myocarditis virus (PMCV)	Probe	TGG TGG AGC GTT CAA	Nylund *et al.* (2018a)
	Forward	AGG GAA CAG GAG GAA GCA GAA	
	Revers	CGT AAT CCG ACA TCA TTT TGT GA	
Salmon gill poxvirus (POX MCP)	Probe	TTA TAC ACC ATC ACA TTT GTG	Nylund, Røed, Blindheim, Trösse, and Andersen (2021)
	Forward	CAG AGG TTT TTC ATA CGC CAG AA	
	Revers	GAG GTC ACG GTG ATG ACA GAA C	
Infectious pancreatic necrosis virus (IPNV)	Probe	TCT TGG CCC CGT TCA TT	Watanabe *et al.* (2006)
	Forward	ACC CCA GGG TCT CCA GTC	
	Revers	GGA TGG GAG GTC GAT CTC GTA	
Paranucleospora theridion (Nuc)	Probe	TTG GCG AAG AAT GAA A	Nylund, Nylund, Watanabe, Arnesen, and Karlsbakk (2010)
	Forward	CGG ACA GGG AGC ATG GTA TAG	
	Revers	GGT CCA GGT TGG GTC TTG AG	
Paramoeba perurans (Pperu)	Probe	CTG GTT CTT TCG RGA GC	Nylund *et al.* (2018b)
	Forward	GAT AAC CGT GGT AAA TCT AGA GCT AAT A	
	Revers	TGG CAT TGG CTT TTG AAT CT	
Ichthyobodo spp. (Costia)	Probe	TCC ACG ACT GCA AAC GAT GAC G	Isaksen, Karlsbakk, Repstad, and Nylund (2012)
	Forward	ACG AAC TTA TGC GAA GGC A	
	Revers	TGA GTA TTC ACT YCC GAT CCA T	
X cell parasite (PER)	Probe	AGC GTT GAG CGG AT	A. Nylund, pers. com.
	Forward	CCC TGC TAA ATA GTA TGC GGT ATA CA	
	Revers	ACC TTC AAA ATA AGA ACA ATC AGC AA	
Tetracapsuloides bryosalmonae (PKX)	Probe	TGT TGT TAG GAT ATT TTC C	A. Nylund, pers. com.
	Forward	CAA GAT CGC GCC CTA TCA AT	
	Revers	CGT CAC CCG TTA CAA CCT TGT	
Parvicapsula pseudobranchicola (Parvi)	Probe	CCG TAT TGC TGT CTT TGA	Nylund *et al.* (2011)
	Forward	TCG TAG TCG GAT GAC AAG AAC GT	
	Revers	AAA CAC CCC GCA CTG CAT	
Perkinsela-like symbiont sp. (Perk)	Probe	CGA AAG CTG AGG CTG T	Røed (2016)
	Forward	GGC ACT GCT CCC CTT CAA C	
	Revers	CGA ACG TAC TTC CCC ATG A	
Candidatus Branchiomonas cysticola (Epit)	Probe	ACT TAG CGA AAG TTA AGC	Nylund *et al.* (2018a)
	Forward	GAG TAA TAC ATC GGA ACG TGT CTA GTG	
	Revers	CTT TCC TCT CCC AAG CTT ATG C	
Candidatus Syngnamydia salmonis (SCh)	Probe	TCC TTC GGG ACC TTA C	Nylund *et al.* (2015)
	Forward	GGG TAG CCC GAT ATC TTC AAA GT	
	Revers	CCC ATG AGC CGC TCT CTC T	
Tenacibaculum spp.	Probe	TTT CAA TAC ATA CAC CTC AGC	Småge *et al.* (2017)

(TB-tuf)	Forward	AGT GTG ACG TCC ACC TT	
	Revers	CTG TAA GCC AGG TTC TGT	
Candidatus Piscichlamydia salmonis (PCh)	Probe	CAA AAC TGC TAG ACT AGA GT	Nylund *et al.* (2008)
	Forward	TCA CCC CCA GGC TGC TT	
	Revers	GAA TTC CAT TTC CCC CTC TTG	
Tenacibaculum maritimum (Tmar)	Probe	TCA TTC AGA CCA GGA GT	Frisch *et al.* (2018)
	Forward	AGA GCA ATT TAC TTC AAC TC	
	Revers	GTA GCA ATT AAG TCT AAT TTA CC	
Atlantic salmon elongation factor (EF1A)	Probe	ATC GGT GGT ATT GGA AC	Olsvik *et al.* (2005)
	Forward	CCC CTC CAG GAC GTT TAC AAA	
	Revers	CAC ACG GCC CAC AGG TAC A	

2.5.3 Efficacy test of real-time RT-PCR assay

All of the assays have been optimized and efficacy tested in advance (table 2.5). This test is done by analyzing a 1:10 dilution series (10^0-10^8) with a template of known concentration in triplicate. The Ct-values at each triplicate is then plotted in a standard curve to calculate the slope and the regression number (R_2) for the assay. Further, the efficacy value (E) is calculated by using the formula (1):

(1) $E = 10^{(-1)/slope}$ (Pfaffl, 2004)

2.6 Density

Density indicates the severity of infection by a particular pathogen in each fish. In this study, density is used to illustrate the amount of specific RNA from one particular pathogen in the sample (volume) that is analyzed. The density is visualized by normalized expression (NE) and as reversed Ct-values.

2.6.2 Normalization of expression values

The analysis results from real-time RT-PCR were normalized against the reference gene EL1A. This is to correct any differences there might be in the amount of tissue at sampling. The normalized expression values (NE) were calculated by using the formula (2):

(2) $NE_{Gill\ tissue} = \frac{(E_{ref})^{Ct\ referance\ gene}}{(E_{target})^{Ct\ targen\ gene}}$ (Simon, 2003)

Further, the normalized expression values were transformed into NE-fold, and to better illustrate the amount of pathogen at each sampling, the data was Log2 transformed (Andersen,

Hodneland, & Nylund, 2010). This was done by dividing the NE-values on the lowest NE-value, as the formula (3):

(3) $NE_{fold} = \frac{NE}{NE_{min}}$

2.6.3 Reversed Ct-values

Reversed Ct-values is a better way to visualize the Ct-values from the analysis. In this method high density is visualized by high values (low Ct-values), and low values (high Ct-values) indicate low density. Reversed Ct-values are calculated by using the formula (4):

(4) $Density = 40 - Ct\ value$

2.7 Prevalence

Prevalence is the amount of a population, given in precent, that is infected by a particular pathogen. It will indicate the occurrence of the microparasite in the studied population. When analyzing 30 fish in a studied population, the prevalence distinguished will be 10 % of the population with 95 % confidence interval. The prevalence is calculated by the formula (5):

(5) $Prevalence = \frac{Number\ of\ positive\ samples}{Total\ number\ of\ samples}\ x\ 100$

2.8 Diversity index

Diversity index is used as a method to describe how many different microparasites is present in one individually fish and in one population. The index is expressed with values from 1-10. Each sampling was analyzed for different number of assays, and therefore the number of pathogens analyzed for will vary within the groups. The diversity index is calculated by the formula (6):

(6) $Diversity\ inde = \frac{Number\ of\ pathogen\ present}{Number\ of\ pathogen\ analyzed\ for}\ x\ 10$

2.9 Statistics

When studying the changes in density of the pathogens in the different groups, NE-fold values from positive individuals were used to perform statistical analysis. The nonparametric Kruskal-Wallis test were used as the statistical test in this study since the NE-fold values are not normally

disturbed, followed by Dunn's multiple comparisons test to compare the mean rank of each group with the mean rank of every other group.

The confidence level for the p-value which indicates significant result in the different statistical analysis were set to $P < 0.05$. The p-values are given in the results as * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, and **** = $P \leq 0.0001$. The adjusted P-values are given in the appendix. To perform all the statistical analysis and to create the graphs and figures the program GraphPad Prism 9 for macOS, version 9.1.0 was used.

3 Results

3.1 Challenge

3.1.1 Abiotic factors

All the environmental factors were held relatively constant throughout the challenge. The water temperature was set to 16 °C (figure 3.1) and the salinity was set to 34 ‰ (figure 3.2) after an acclimatization period of three days. The fish were held at a 12:12 light regime and the water flow at 400 liters/hour, which was regulated if needed (by the staff at ILAB) throughout the challenge based on increased biomass in the tanks.

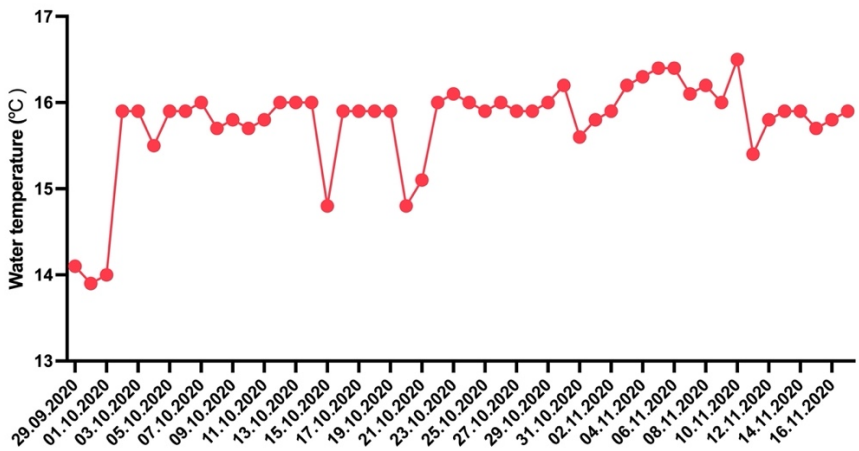

Figure 3.1 Temperature registered daily by the staff at ILAB during the period of challenge (29.09.2020-17.11.2020).

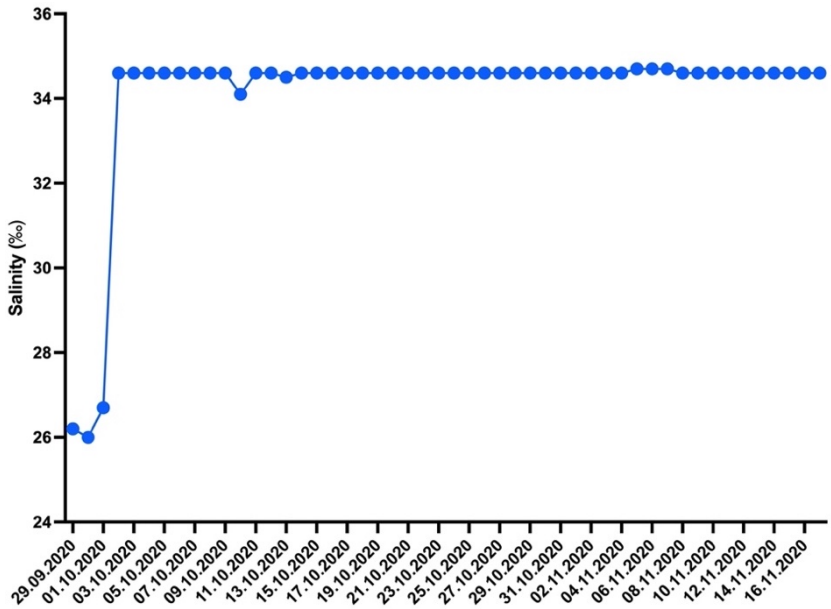

Figure 3.2 Salinity registered daily by the staff at ILAB during the period of challenge (29.09.2020-17.11.2020).

3.1.2 Biotic factors

There was no registration of fish dying during the challenge. The average weight and length increased slightly during the challenge (table 3.1, figure 3.3).

At the 1st sampling (17 dpi) few external changes were observed. There was some fish with scale loss (in the anterior part of the fish), one fish with ulcers at the belly, and one fish with some blood on the snout/mouth area. There was no observation of external changes at the 2nd sampling (25 dpi). At the third sampling (27 dpi) there was observation of some ulcers at the belly and the snout/mouth area. There were more external changes at the fourth sampling (32 dpi). In tank 1, there was six fish with ulcers of different size at the belly, four fish with ulcers on the mouth, one fish had deformity of the operculum, and one fish with an infection on the lower jaw. In tank 2 there was two fish with ulcers on the snout/mouth area, and one fish with four ulcers at the belly. In tank 3 there was five fish with ulcers of different size at the belly, one fish had bleeding in the eye area, and one fish had eye cataract on the left eye. In tank 4, there was seven fish with different ulcers of different sizes. In tank 5 there was five fish with ulcers of different size on the belly and three fish had eye cataract (one at the left eye, and two at the right eye). In tank 7 there was one fish with ulcers at the belly. In tank 8 there was six fish with ulcers of different size on the belly and one fish had ulcers at the snout/mouth area.

Table 3.1 Average weight and length for each tank at all four sampling (19, 25, 27 and 32 dpi). N = 5 fish at 1st sampling, N = 2 fish at 2nd sampling, N = 10 fish at 3rd sampling, N = 22 fish from tank 1, 5 and 7, N = 21 fish from tank 6 and 8, N = 20 fish from tank 3 and 4, N = 19 fish from tank 2 at 4th sampling.

	1st sampling (19 dpi)		**2nd sampling (25 dpi)**		**3rd sampling (27 dpi)**		**4th sampling (32 dpi)**	
	Weight	**Length**	**Weight**	**Length**	**Weight**	**Length**	**Weight**	**Length**
Tank 1	133,8	21,6	111,2	20,5	131,0	22,2	148,2	23,0
Tank 2	152,0	22,9	124,9	21,7	164,4	23,6	163,8	23,6
Tank 3	121,9	21,6	155,5	23,0	146,8	23,1	157,4	23,4
Tank 4	126,8	21,7	173,2	25,8	128,3	22,1	151,1	23,1
Tank 5	152,3	23,3	168,9	24,4	161,8	23,9	163,3	23,8
Tank 6	162,1	23,0	149,8	22,8	147,9	22,8	167,4	23,5
Tank 7	117,1	21,3	157,7	23,9	149,0	22,6	170,7	24,0
Tank 8	143,8	22,6	171,0	24,1	163,8	23,7	165,2	23,6

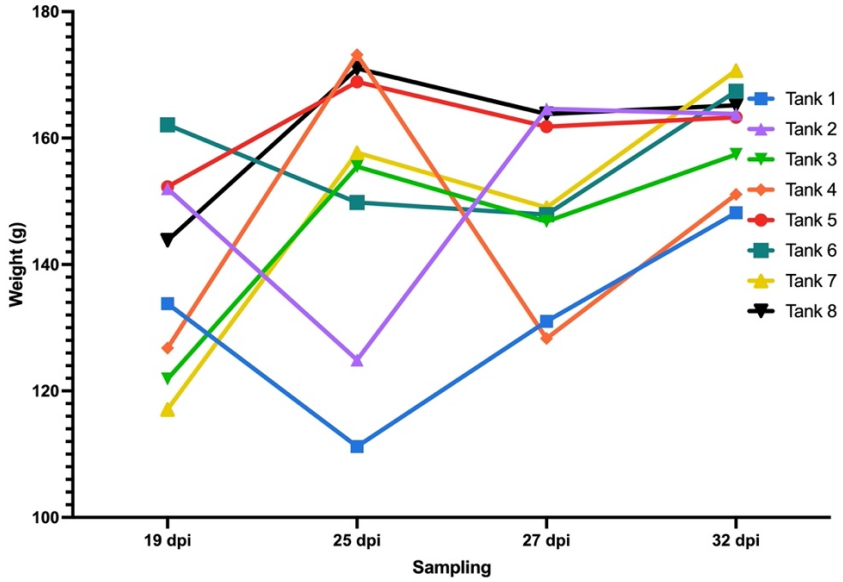

Figure 3.3 Average weight for each tank at all four sampling (19, 25, 27 and 32 dpi). N = 5 fish at 19 dpi, N = 2 fish at 25 dpi, N = 10 fish at 27 dpi, N = 22 fish from tank 1, 5 and 7, N = 21 fish from tank 6 and 8, N = 20 fish from tank 3 and 4, N = 19 fish from tank 2 at 32 dpi.

3.1.3 Gill score

Lesions at the gills were observed as white patches on the gill surface. There were no severe gill lesions during the samplings (figure 3.4). At 19 dpi the lowest mean gill score was 0.0 (tank 5 and 8) and the highest mean gill score was 0.2 (tank 4). Six days later at 25 dpi, the lowest mean gill score was 0.0 (tank 5 and 8), and the highest mean gill score was 0.1 (tank 4). At 27 dpi the lowest mean gill score was 0.0 (tank 5 and 8), and the highest mean gill score was 0.1 (tank 1). The sampling at 32 dpi, the lowest mean gill score was 0.1 (tank 1), and the highest mean gill score was 0.2 (tank 4).

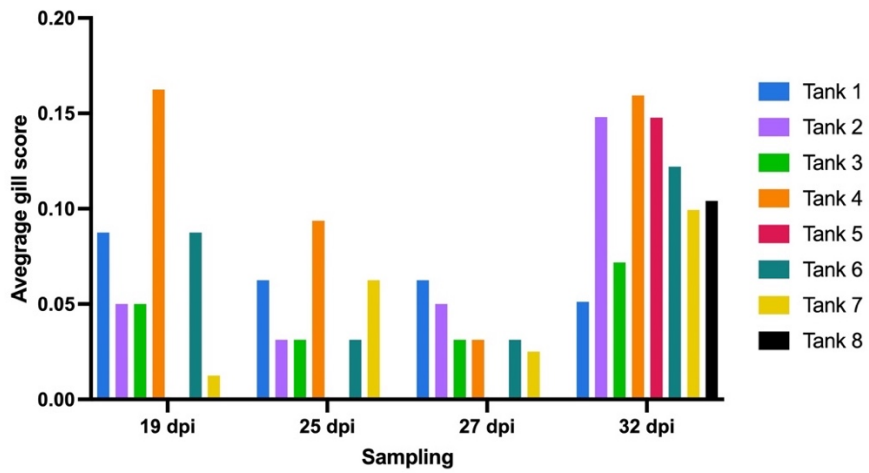

Figure 3.4 Average gill score for each tank during the challenge at 19, 25, 27, 32 dpi. N = 5 fish at 19 dpi, N = 2 fish at 25 dpi, N = 10 fish at 27 dpi, N = 22 fish from tank 1, 5 and 7, N = 21 fish from tank 6 and 8, N = 20 fish from tank 3 and 4, N = 19 fish from tank 2 at 32 dpi.

The number of gill surfaces scored from each fish at all samplings were 16. At 27 dpi the total number of gill surfaces was 272 (N = 17) for each tank. The highest number of gill surfaces with registered gill score is 20 (tank 4), and the lowest number of gill surfaces with registered gill score is 0 (tank 7 and 8) (figure 3.5). Gill score higher than score 1 was registered only in tank 6 (NM-H20) and tank 4 (ST19/15Pp) (only score 2).

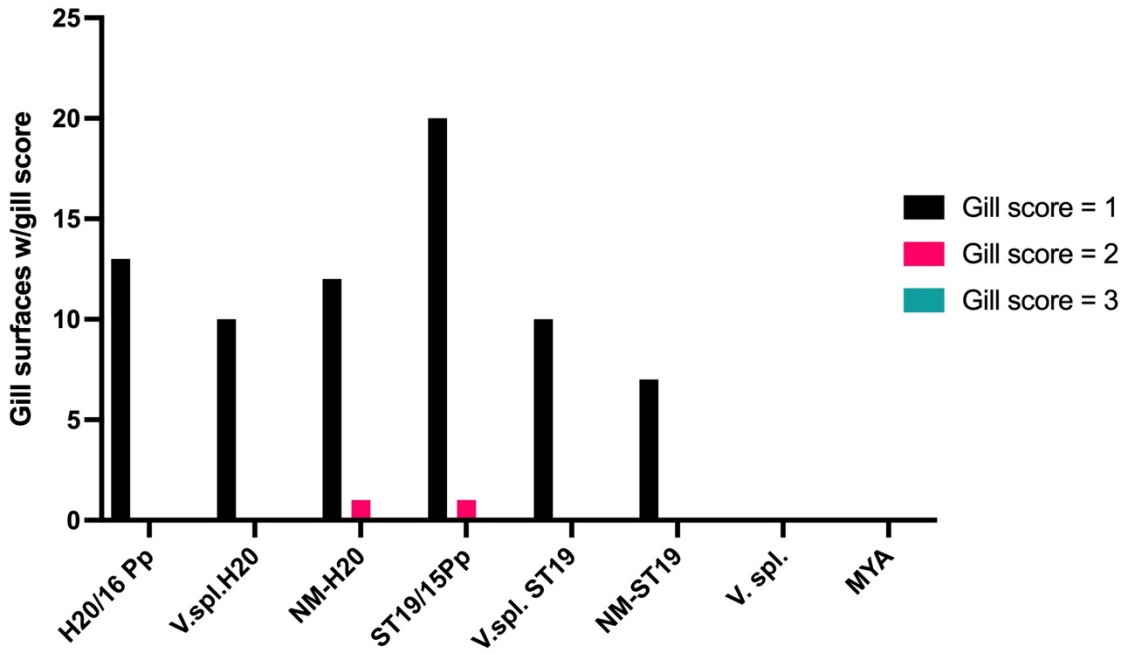

Figure 3.5 Number of gill surfaces with registered gill score for each of the groups at 27 dpi. N = 17 for each group (272 gill surfaces). H20/16Pp = Tank 2 (Clone H20/16Pp grown in normal media), V.spl.H20 = Tank 1 (Clone H20/16Pp grown in pure culture of *V. splendidus*), NM-H20 = Tank 6 (Normal media containing microbiota obtained from the gills of farmed salmon during first isolation H20/16Pp in 2016), ST19/15Pp = Tank 4 (Clone ST19/15Pp grown in normal media), V.spl. ST19 = Tank 3 (Clone ST19/15Pp grown in pure culture of *V. splendidus*), NM-ST19 = Tank 7 (Normal media containing microbiota obtained from the gills of farmed salmon during first isolation ST19/15Pp in 2015), V.spl. = Tank 5 (Cloned culture of *V. splendidus*), MYA = Tank 8 (Control; exposed to bacteria- and amoeba-free Malt Yeats media).

At 32 dpi the total number of scored gill surfaces varied for each tank. N = 22 fish (352 gill surfaces) from tank 1, 5 and 7, N = 21 fish (336 gill surfaces) from tank 6 and 8, N = 20 fish (320 gill surfaces) from tank 3 and 4, N = 19 fish (380 gill surfaces) from tank 2. The highest number of gill surfaces with registered gill score was 42 (tank 4), and the lowest number of gill surfaces with registered gill score was 15 (tank 1) (figure 3.6). Illustrated in figure 3.6, gill score higher than score 1 was registered in all tanks (only score 2).

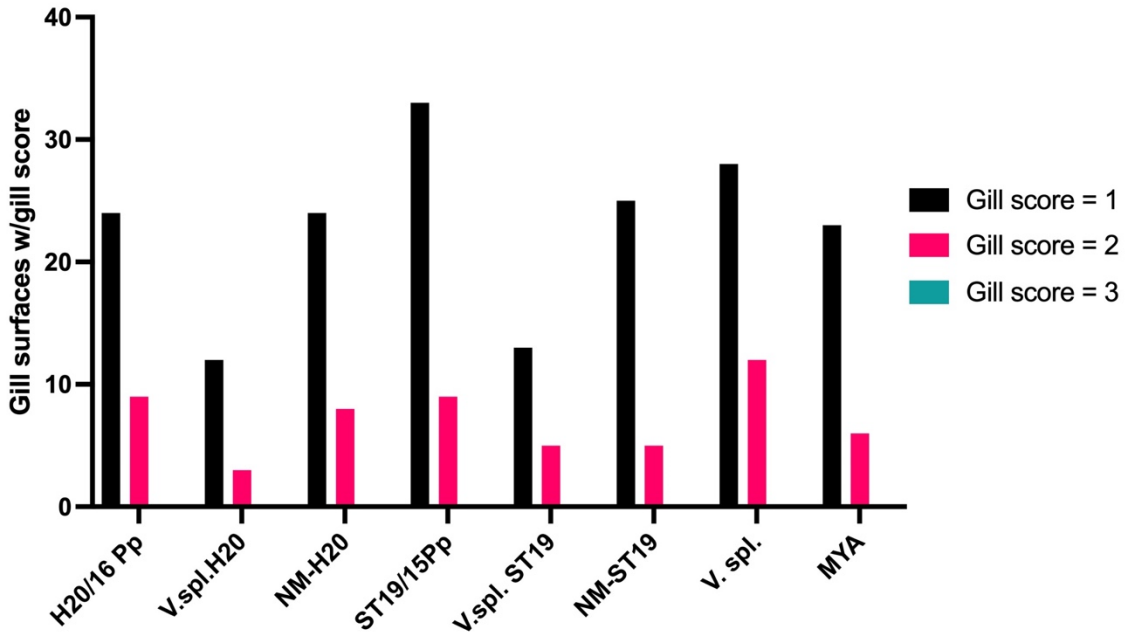

Figure 3.6 Number of gill surfaces with registered gill score for each of the groups at 32 dpi. N = 22 fish (352 gill surfaces) from tank 1, 5 and 7, N = 21 fish (336 gill surfaces) from tank 6 and 8, N = 20 fish (320 gill surfaces) from tank 3 and 4, N = 19 fish (380 gill surfaces) from tank 2. H20/16Pp = Tank 2 (Clone H20/16Pp grown in normal media), V.spl.H20 = Tank 1 (Clone H20/16Pp grown in pure culture of *V. splendidus*), NM-H20 = Tank 6 (Normal media containing microbiota obtained from the gills of farmed salmon during first isolation H20/16Pp in 2016), ST19/15Pp = Tank 4 (Clone ST19/15Pp grown in normal media), V.spl. ST19 = Tank 3 (Clone ST19/15Pp grown in pure culture of *V. splendidus*), NM-ST19 = Tank 7 (Normal media containing microbiota obtained from the gills of farmed salmon during first isolation ST19/15Pp in 2015), V.spl. = Tank 5 (Cloned culture of *V. splendidus*), MYA = Tank 8 (Control; exposed to bacteria- and amoeba-free Malt Yeats media).

3.1.4 Real time RT-PCR

During the challenge experiment, 303 samples of gill tissue was analyzed for *P. perurans*, *Perkinsela*-like symbiont and *Candidatus* Syngnamydia salmonis. Most of the samples analyzed for *P. perurans* was negative (table 3.2). At 19 dpi the prevalence of *P. perurans* was 100 % in tank 4 (Clone ST19/15Pp grown in normal media), 66.7 % in tank 6 (Normal media from H20/16Pp), 20 % in tank 1 and 0 % (tank 2, 3 5, 7 and 8). At 25 dpi the prevalence of *P. perurans* was 50% in tank 4 and 0 % in tank 1, 2, 3, 5, 6, 7 and 8. The prevalence of *P. perurans* at 27 dpi was 70 % in tank 4 and 0 % in tank 1, 2, 3, 5, 6, 7, and 8. At 32 dpi the prevalence of *P. perurans* was 0 % for all of the tanks. The rest of the Ct- values and prevalence for the assays analyzed for is given in the appendix.

Table 3.2 Average and range of Ct-values and prevalence in percent of *P. perurans* (Pperu assay) at each sampling (19, 25, 27 and 32 dpi). At 19 dpi N = 10 individuals per tank, 25 dpi N = 2 individuals per tank, 27 dpi N = 10 individuals per tank and at 32 dpi N = N = 22 fish from tank 1, 5 and 7, N = 21 fish from tank 6 and 8, N = 20 fish from tank 3 and 4, N = 19 fish from tank 2

	19 dpi			**25 dpi**			**27 dpi**			**32 dpi**		
	Ct-values		Prevalence	Ct-values		Prevalence	Ct-values		Prevalence	Ct-values		Prevalence
	Average	Range	%	Average	Range	%	Average	Range	%	Average	Range	%
V.spl.H20	35.9	35.9 – 35.9	10	Neg	Neg	0	Neg	Neg	0	Neg	Neg	0
H20/16Pp	Neg	Neg	0	Neg	Neg	0	Neg	Neg	0	Neg	neg	0
V.spl. ST19	Neg	Neg	0	Neg	Neg	0	Neg	Neg	0	Neg	Neg	0
ST19/15Pp	27.7	19.5 – 37.7.	100	30.4	30.4 – 30.4	50	31.5	28.4 – 34.6	70	Neg	Neg	0
V.spl.	Neg	Neg	0	Neg	Neg	0	Neg	Neg	0	Neg	Neg	0
NM-H20	34.9	33.6 – 36.2	40	Neg	Neg	0	Neg	Neg	0	Neg	Neg	0
NM-ST19	Neg	Neg	0	Neg	Neg	0	Neg	Neg	0	Neg	Neg	0
MYA	Neg	Neg	0	Neg	Neg	0	Neg	Neg	0	Neg	Neg	0

3.2 Freshwater treatment against AGD

At the first sampling date, the average weight and length of the fish (*Salmo salar*) was 2448 ± 492 grams and 60 ± 3 cm respectively. There were four mobile lice present at four fish at the sampling prior to the treatment. Eight of the fish had pale gills (figure 3.7 D), two fish had cataract (figure 3.7 A), one fish had eye bleeding (figure 3.7 B), and one fish had ulcer at the left side (figure 3.7 C). The rest of the fish sampled before treatment had little visible pathology. After the freshwater treatment and sampling of the treated group, the average weight and length of the treated fish was 2621 ± 677 grams and 59 ± 4 cm respectively. Most of the pathology observed within the treated group was eye bleeding. Eye bleeding was observed in eight fish, three fish had pale gills, one fish had cataract, one fish had erosion of the second gill arch and one fish was missing the dorsal fin. The average weight and length of the untreated fish was 2684 ± 641 grams and 60 ± 4 cm respectively. The pathology for the untreated group was mostly cataract and eye bleeding, where six fish had cataract and seven had eye bleeding. Three fish had pale gills.

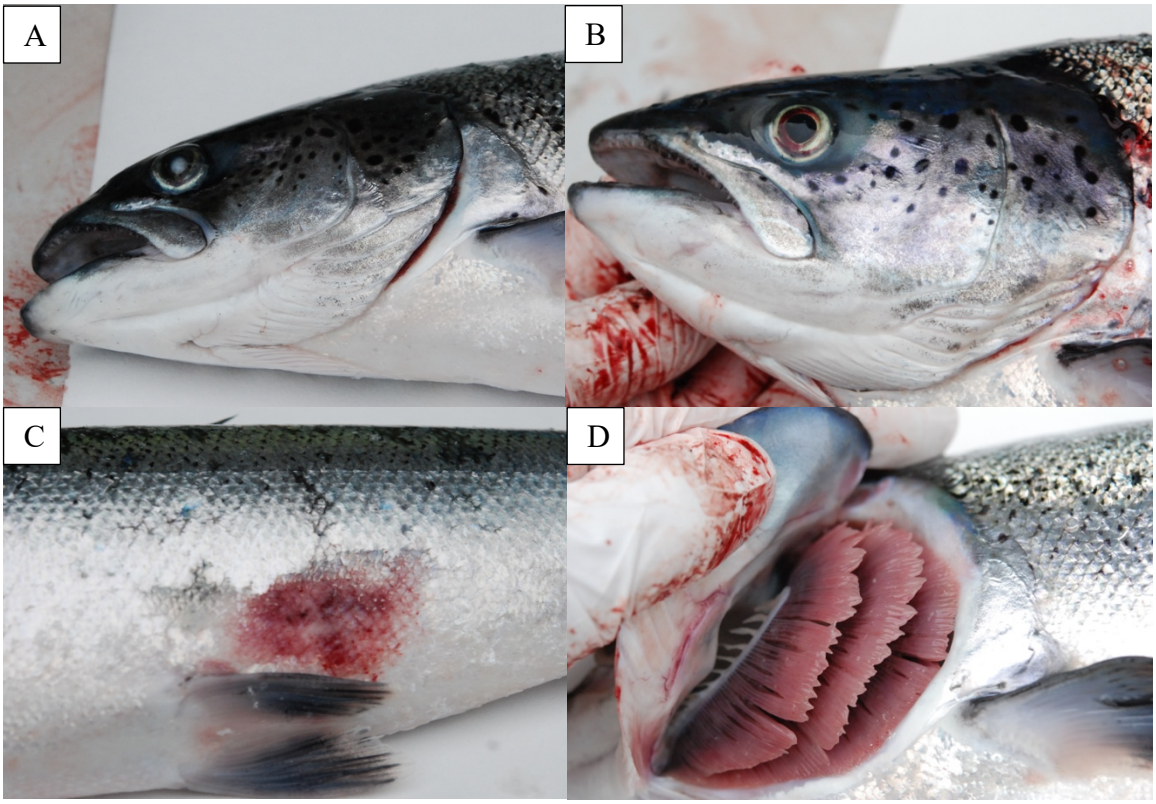

Figure 3.7 Pathology observed at sampling before the freshwater treatment at locality LB. **A**: cataract, **B**: eye bleeding, **C**: ulcer close to the pelvic fin, **D**: pale gills.

The average sea temperature during the ten weeks prior to treatment was 15.9 °C, and the average number of mobile lice per fish was 1.8. The temperature was 14.9 °C at the day of treatment, and the number of mobile lice per fish was 0.07 (figure 3.8). During the sampling post treatment, there was no presence of mobile lice.

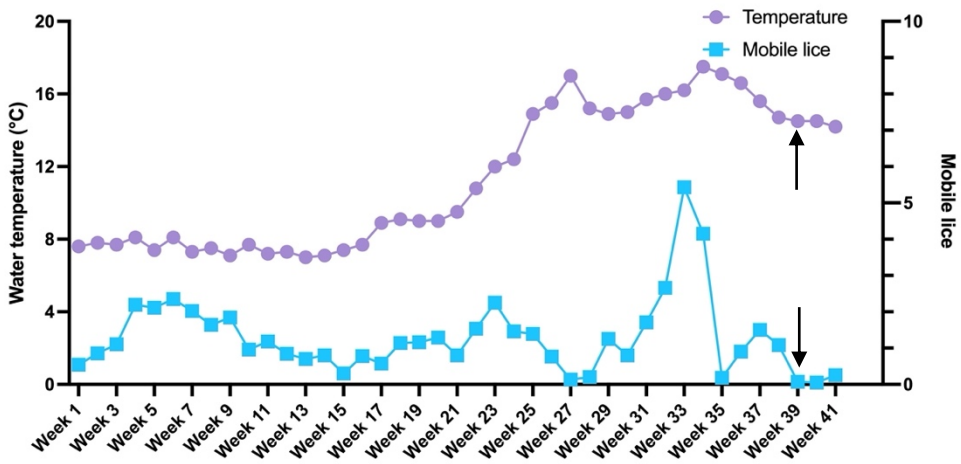

Figure 3.8 Temperature (purple) and number of mobile lice (blue) from week 1 in 2020 until week 41 when all the fish at locality LB were sent to slaughter and processing. The first sampling and treatment were performed during week 39 (arrow), and the sampling post treatment were performed in week 40. Data obtained from barentswatch.no.

Prior to the treatment, the mortality each day for the last fourteen days was 100-300 fish. 1500 fish died during the treatment, which accounts for 1.05 % of the biomass in the treated cage. As for the fish that died during treatment, no significant gross pathology was observed. The mortality rate for the treated cage was reduced after the treatment to 10-60 fish each day until week 41. For the untreated cage the mortality rate was approximately 150-200 fish each day during the same period of time.

3.2.1 Gill score

During the sampling, scoring of gills was conducted. Lesions on the gills was observed as white patches with varying severity. Example of lesions/patches on the gills is illustrated in figure 3.9. Some of the individuals had more severe lesions on the gills, and others had no or little lesions on the gills. Within the sampling group "before treatment", "treated" and "untreated", there is small changes of the gill score (figure 3.10).

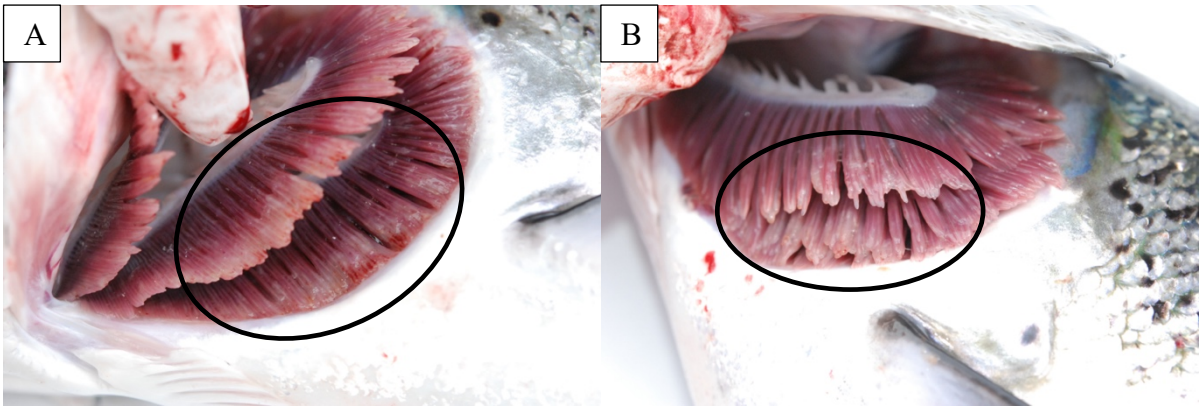

Figure 3.9 Infected gills with clear lesions/patches. The circles indicate area with lesions/patches at the gills. **A**: gills form fish sampled before treatment. **B**: gills form fish sampled after treatment form the treated group.

The four gill arches at the left side were scored according to table 2.2, front and back of each gill arch. This gives 8 gill surfaces per fish. Gill lesions was observed within all three groups when sampling. The average gill score before treatment, for treated group and untreated group was 1.1 ± 0.8, 1.0 ± 0.9 and 1.0 ± 0.7 respectively (figure 3.10). The gill arch with the highest average gill score before treatment was the front side of the third gill arch, this was also the case for the treated group. As for the untreated group, the gill arch with the highest average gill score was the front side of the second gill arch.

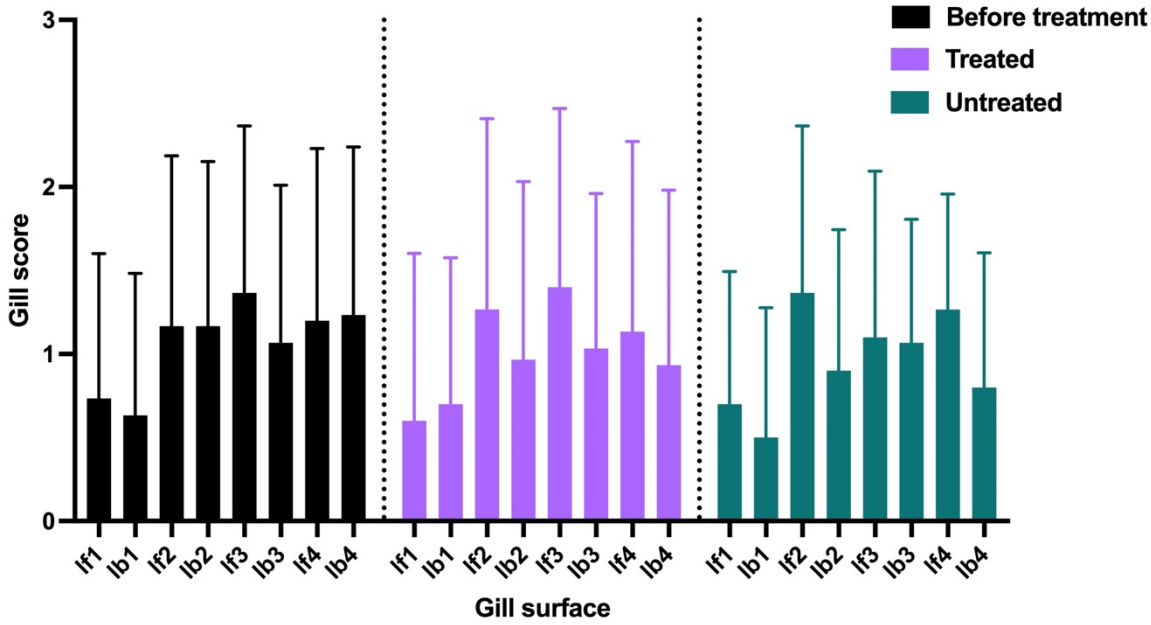

Figure 3.10 Average gill score of each side of the gill arches with standard deviation for each sampling. N = 30 fish for each group which implies 30 gill surfaces for each column. Lf = left side of the fish, front side of the gill arch, lb = left side of the fish, back side of the gill arch, number = number of gill arch (1-4).

The highest average gill score for one individual fish prior to the freshwater treatment was 2.9, for the treated group the highest average score was 3.6, and for the untreated group the highest average score was 2.3 (figure 3.10). The average gill scores within the different groups are slightly lower for the treated group than before treatment, with values 1.01 and 1.08 respectively, which is presented in figure 3.11. Also, the average gill scores for the untreated group are slightly lower than before treatment and treated group.

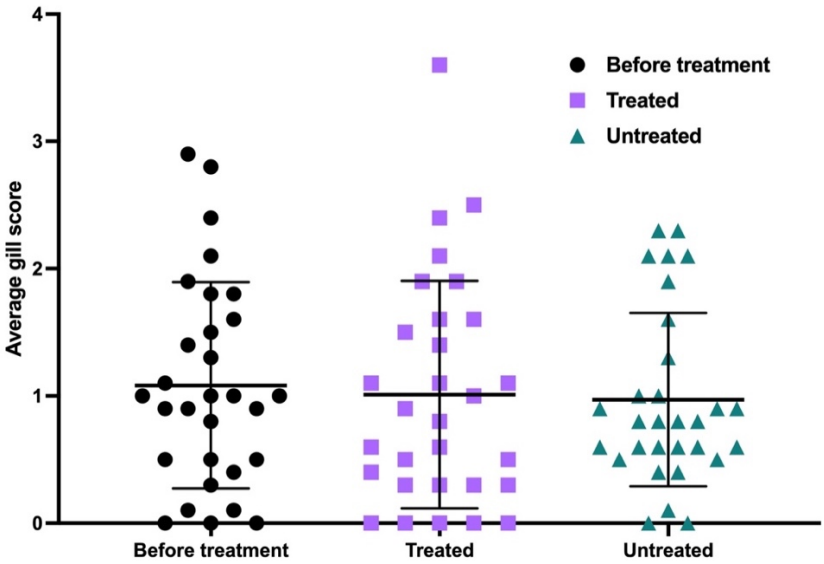

Figure 3.11 Average gill score within each group. Each point represents one individual fish. The thicker line in the middle for each group indicates the average of the gill score, and the thinner and smaller line across indicates the standard deviation.

The maximum number of free, or non-affected, gill surfaces for each individual is 8, as only the left side of the gills were scored. The amount of free gill surfaces is slightly higher for the treated group than before treatment (figure 3.12). Before treatment, only three individuals had 8 free gill surfaces compared to five individuals within the treated group. The untreated group had only two individuals with 8 free gill surfaces. The number of fish with no free gill surfaces before treatment was nine individuals compared to seven individuals for both treated and untreated group. Also, the treated group had more individuals with 4 or more free gill surfaces compared to the group scored before treatment and the untreated group.

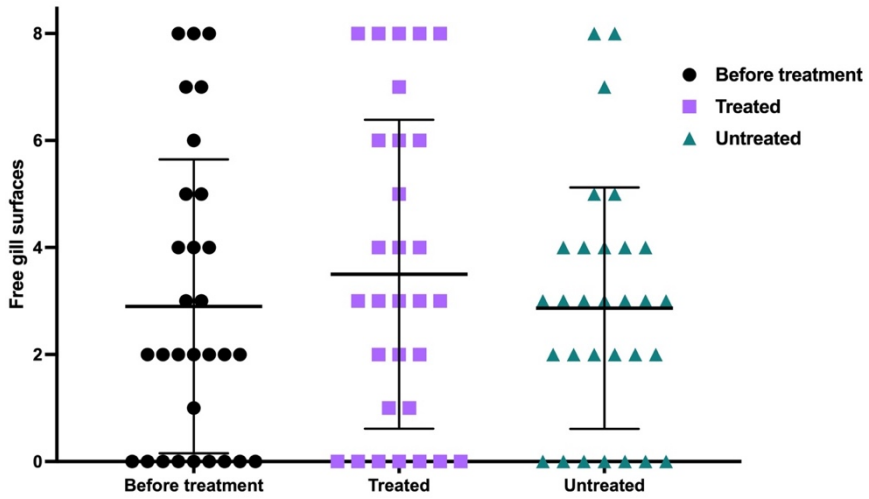

Figure 3.12 Number of free gill surfaces for each sampling. Each point represents one individual fish. The thicker line in the middle for each group indicates the average amount of free gill surfaces, and the thinner and smaller line across indicates the standard deviation.

3.2.2 Real time RT-PCR and mapping of microparasites

Of all the 118 samples from the gills that was analyzed, there was no prevalence of Salmonid alphavirus (SAV), Infectious salmon anemia virus (ISAV) and Infectious pancreatic necrosis virus (IPNV) (figure 3.13). There is a low prevalence of *Parvicapsula pseudobranchicola* (Parvi), *Tenacibaculum* spp. (TB-tuf) and *Candidatus* Piscichlamydia salmonis (Ca. P. s.), and the Ct-values are relatively high (appendix). The prevalence of *Piscine orthoreovirus* 1 (PRV1), Piscine myocarditis virus (PMCV), Salmon gill poxvirus (SGPV), *Paranucleospora theridion* (Nuc) and *Candidatus* Branchiomonas cysticola (Ca. B. c.) are 100 % within all the sampling groups. For some of the pathogens, the prevalence was reduced for the treated group and/or the group that died during treatment, such as *Paramoeba perurans* (Pperu), *Ichthyobodo* spp. (Costia) and *Candidatus* Syngnamydia salmonis (Ca. S. s.), as illustrated in figure 3.13.

Table 3.3 Prevalence in percent of the different pathogens at the gills that were analyzed for within the four groups. SAV = Salmonid alphavirus, ISAV = Infectious salmon anemia virus, PRV1 = *Piscine orthoreovirus* 1, PMCV = Piscine myocarditis virus, SGPV = Salmon gill poxvirus, IPNV = Infectious pancreatic necrosis virus, Nuc = *Paranucleospora theridion*, Pperu = *Paramoeba perurans,* Costia = *Ichthyobodo* spp., Parvi = *Parvicapsula pseudobranchicola*, Ca. B. c. = *Candidatus* Branchiomonas cysticola, Ca. S. s. = *Candidatus* Syngnamydia salmonis, TB-tuf = *Tenacibaculum* spp., Ca. P. s. = *Candidatus* Piscichlamydia salmonis.

	Before treatment		**During treatment**		**Treated**		**Untreated**	
	Number of positive individuals	Prevalence (%)	Number of positive individuals	Prevalence (%)	Number of positive individuals	Prevalence (%)	Number of positive individuals	Prevalence (%)
SAV	0 of 30	0.0	0 of 28	0.0	0 of 30	0.0	0 of 3	0.0
ISAV	0 of 30	0.0	0 of 28	0.0	0 of 30	0.0	0 of 30	0.0
PRV1	30 of 30	100.0	28 of 28	100.0	30 of 30	100.0	30 of 30	100.0
PMCV	30 of 30	100.0	28 of 28	100.0	30 of 30	100.0	30 of 30	100.0
SGPV	30 of 30	100.0	28 of 28	100.0	28 of 30	93.3	30 of 30	100.0
IPNV	0 of 30	0.0	0 of 28	0.0	0 of 30	0.0	0 of 30	0.0
Nuc	30 of 30	100.0	28 of 28	100.0	30 of 30	100.0	30 of 30	100.0
Pperu	30 of 30	100.0	21 of 28	75.0	10 of 30	33.3	30 of 30	100.0
Costia	29 of 30	96.7	17 of 28	60.7	6 of 30	20.0	30 of 30	100.0
Parvi	0 of 30	0.0	5 of 28	17.9	2 of 30	6.7	2 of 30	6.7
Ca. B. c.	30 of 30	100.0	28 of 28	100.0	30 of 30	100.0	30 of 30	100.0
Ca. S. s.	30 of 30	100.0	27 of 28	96.4	11 of 30	36.6	30 of 30	100.0
TB-tuf	4 of 30	13.3	7 of 28	25.0	6 of 30	20.0	14 of 30	46.7
Ca. P. s.	3 of 30	10.0	1 of 28	3.6	2 of 30	6.7	3 of 30	10.0

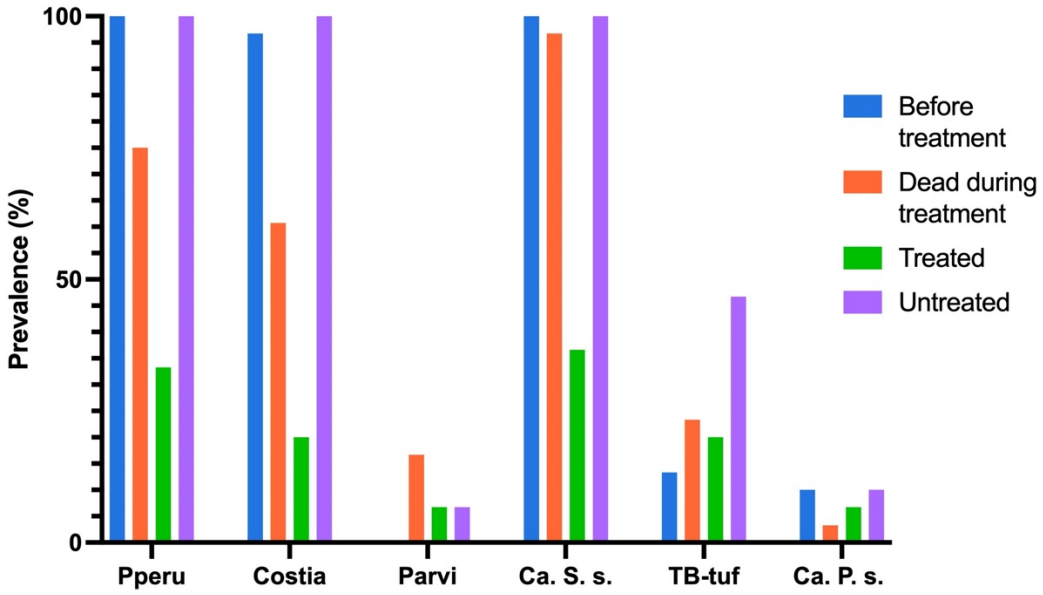

Figure 3.13 Prevalence of pathogens at the gills that changed during the sampling. **Pperu**: 30 of 30 positive individuals before treatment, 21 of 28 positive individuals died during treatment, 10 of 30 positive individuals treated, 30 of 30 positive individuals untreated. **Costia**: 29 of 30 positive individuals before treatment, 17 of 28 positive individuals died during treatment, 6 of 30 positive individuals treated, 30 of 30 positive individuals untreated. **Parvi**: 0 of 30 positive individuals before treatment, 5 of 28 positive individuals died during treatment, 2 of 30 positive individuals treated, 2 of 30 positive individuals untreated. **Ca. S. s**.: 30 of 30 positive individuals before treatment, 27 of 28 positive individuals died during treatment, 11 of 30 positive individuals treated, 29 of

30 positive individuals untreated. **TB-tuf**: 4 of 30 positive individuals before treatment, 7 of 28 positive individuals died during treatment, 6 of 30 positive individuals treated, 14 of 30 positive individuals untreated. **Ca. P. s**.: 3 of 30 positive individuals before treatment, 1 of 28 positive individuals died during treatment, 2 of 30 positive individuals treated, 3 of 30 positive individuals untreated. Pperu = *Paramoeba perurans,* Costia = *Ichthyobodo* spp., Parvi = *Parvicapsula pseudobranchicola*, Ca. S. s. = *Candidatus* Syngnamydia salmonis, TB-tuf = *Tenacibaculum* spp., Ca. P. s. = *Candidatus* Piscichlamydia salmonis.

The prevalence of *P. perurans* at the gills prior to the treatment was 100 %, which were reduced to 33.3 % for the treated group. For the group that died during treatment the prevalence was 75.0 %, and for the untreated group it was 100 %. The density of *P. perurans* for the analyzed gill tissue was significant higher before treatment and for the untreated group compared to the treated group and the group that died during treatment ($P \leq 0.0001$) (figure 3.14). There was no significant difference for "before treatment" vs. "untreated" ($P > 0.9999$) and "dead during treatment" vs. "treated" ($P > 0.9999$).

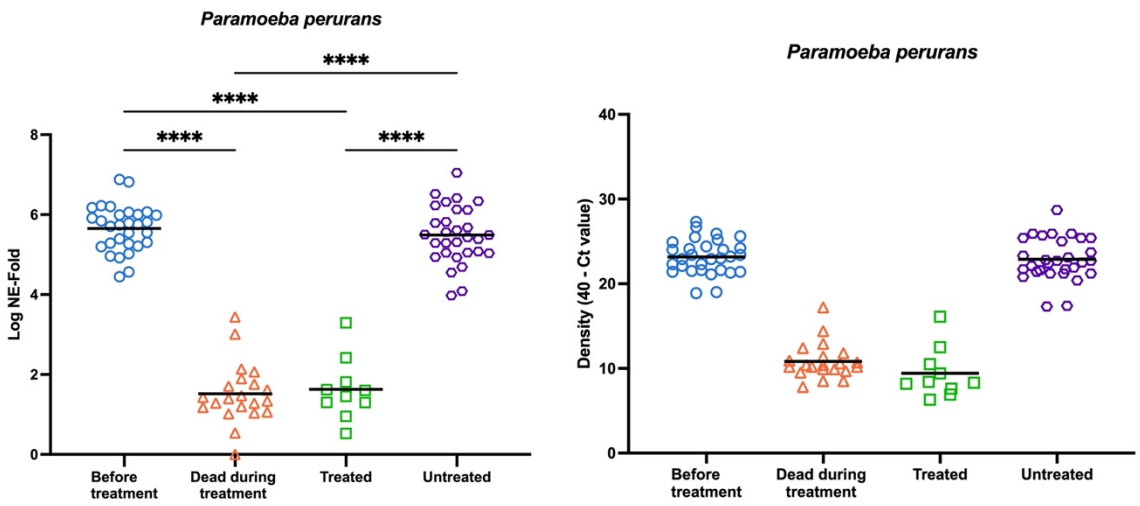

Figure 3.14 Density of *P. perurans* at the gills for all of the groups. Each point represent one individual fish. The data is presented as reversed Ct-values (40 – Ct-value) and Log NE-fold. The black line within each group represent the average Ct- value. N = 30 positive individuals before treatment. N = 21 postitive individuals died during treatement. N = 8 positive individuals treated. N = 30 positive individuals untreated. **** $P \leq 0.0001$.

The prevalence of *Ichthyobodo* spp. was also reduced from 100% before treatment to 20.0 % for the treated group, but for the untreated group there was no reduction as the prevalence was 100 %. The prevalence for the group that died during treatment was 60.7 %. Density of *Ichthyobodo* spp. for the analyzed gill tissue was significant higher before treatment compared to the group that died during treatment ($P = 0.0023$) and for the untreated group compared to the group the died during treatment ($P = 0.0013$) (figure 3.15). There was no significant difference for "before treatment" vs. "treated" ($P = 0.0763$), "before treatment" vs. "untreated"

(P > 0.9999), “dead during treatment” vs. “treated” (P > 0.9999), and “treated” vs. “untreated” (P = 0.0579).

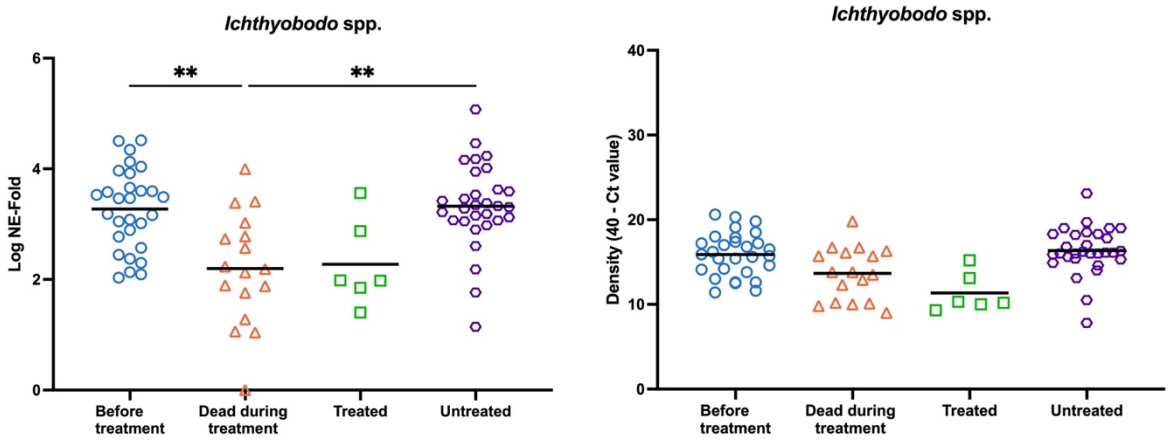

Figure 3.15 Density of *Ichthyobodo* spp. at the gills for all of the groups. Each point represent one individual fish. The data is presented as reversed Ct-values (40 – Ct-value) and Log NE-fold. The black line within each group represent the average Ct- value. N = 29 positive individuals before treatment. N = 17 postitive individuals died during treatement. N = 5 positive individuals treated. N = 30 positive individuals untreated. ** P ≤ 0.01.

For *Candidatus* Syngnamydia salmonis, the prevalence before treatment was 100 % compared to 36.6 % after treatment. The prevalence for the untreated group was 100 %, while it was 96.4 % for the group that died during treatment. The density of *Candidatus* Syngnamydia salmonis for the analyzed gill tissue was significant higher before treatment compared to the group that died during treatment (P ≤ 0.0001), before treatment compared to the treated group (P ≤ 0.0001), for the untreated group compared to the group that died during treatment (P ≤ 0.0001), and for the untreated group compared to the treated group (P ≤ 0.0001) (figure 3.16). There was no significant difference for “before treatment” vs. “untreated” (P > 0.9999), “dead during treatment” vs. “treated” (P > 0.9999).

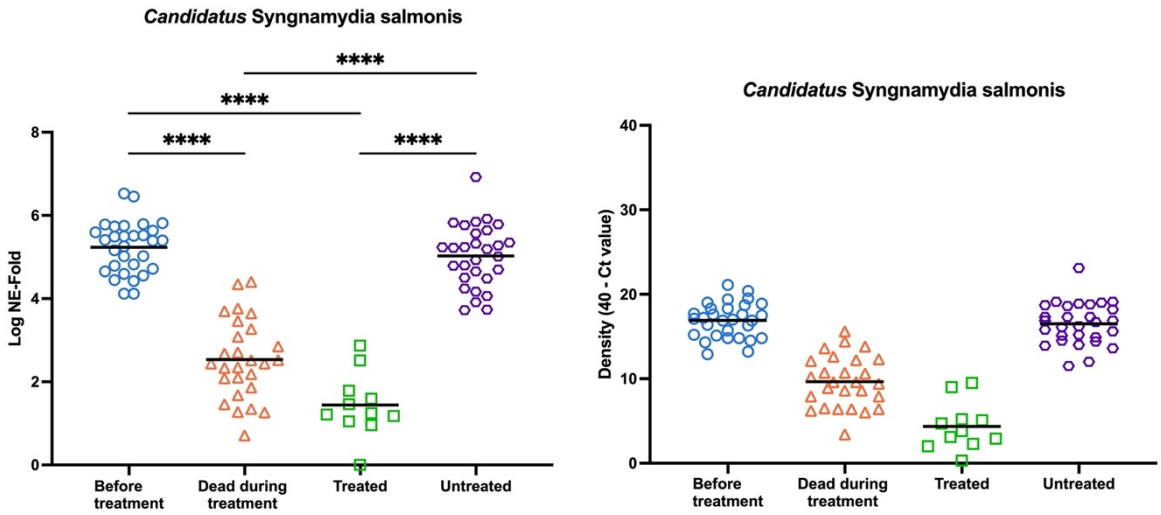

Figure 3.16 Density of *Candidatus* Syngnamydia salmonis at the gills for all of the groups. Each point represent one individual fish. The data is presented as reversed Ct-values (40 – Ct-value) and Log NE-fold. The black line within each group represent the average Ct- value. N = 30 positive individuals before treatment. N = 27 postitive individuals died during treatement. N = 10 positive individuals treated. N = 29 positive individuals untreated. **** $P \leq 0.0001$.

The prevalence of *Candidatus* Branchiomonas cysticola in the analyzed gill tissue was 100 % within all groups. The density in the gills was significant higher for the group that died during treatment compared to before treatment (P = 0.0047), the treated group (P = 0.0003) and the untreated group (P < 0.0001) (figure 3.17). There were no significant differences between the rest of the groups.

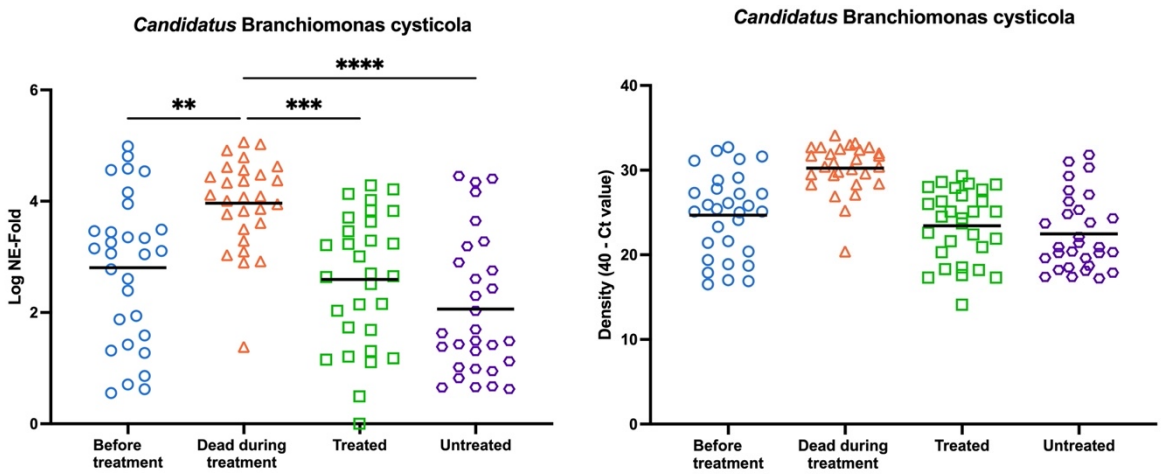

Figure 3.17 Density of *Candidatus* Branchiomonas cysticola at the gills for all of the groups. Each point represent one individual fish. The data is presented as reversed Ct-values (40 – Ct-value) and Log NE-fold. The black line within each group represent the average Ct- value. N = 30 postitive individuals for every group (except "dead during treatment" N = 28). ** $P \leq 0.01$, *** $P \leq 0.001$ and **** $P \leq 0.0001$.

The prevalence of piscine myocarditis virus at the gills was 100 % for all the groups. The density of the virus in the gills was significant higher for the group that died during treatment compared to the group sampled before treatment (P = 0.0001) and for the untreated group compared to the group that died during treatment (P = 0.0025) (figure 3.18). There were no significant differences between "before treatment" vs. "treated" (P = 0.3284), "before treatment" vs. "untreated" (P > 0.9999), "dead during treatment" vs. "treated" (P = 0.1042) and for the "treated" vs. "untreated" (P > 0.9999). In the kidney the prevalence was 100 % for all groups (except before treatment, 93.3 %). The density of the virus in the kidney was significant higher for the group that died during treatment compared to the group sampled before treatment (P = 0.0001), For the group that died during treatment compared to the untreated group (P < 0.0001) and for the treated group compared to the untreated group (P = 0.0074) (figure 3.19). There were no significant differences between "before treatment" vs. "treated" (P = 0.3619), "before treatment" vs. "untreated" (P > 0.9999) and for "dead during treatment" vs. "treated" (P = 0.0927).

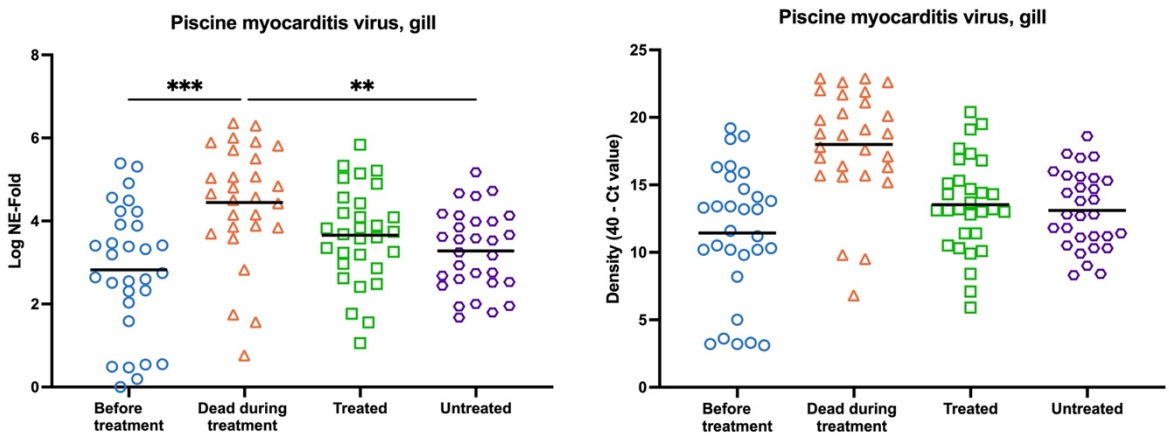

Figure 3.18 Density of Piscine myocarditis virus at the gills for all of the groups. Each point represent one individual fish. The data is presented as reversed Ct-values (40 – Ct-value) and Log NE-fold. The black line within each group represent the average Ct- value. N = 30 positive individuals for every group (except "dead during treatment" N = 28). ** P ≤ 0.01 and *** P ≤ 0.001.

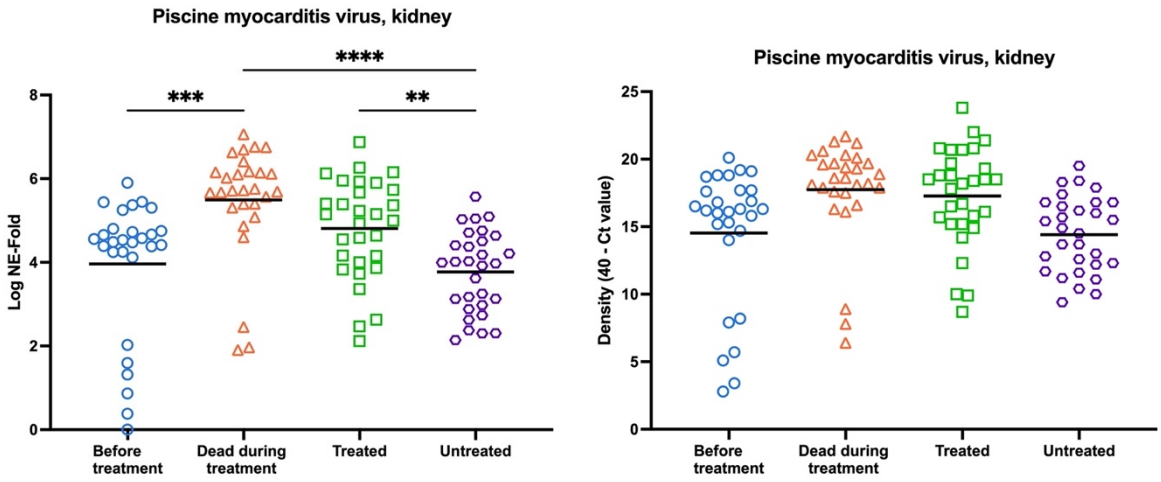

Figure 3.19 Density of Piscine myocarditis virus in the kidney for all of the groups. Each point represent one individual fish. The data is presented as reversed Ct-values (40 – Ct-value) and Log NE-fold. The black line within each group represent the average Ct- value. N = 28 positive individuals before treatment. N = 28 postitive individuals died during treatement. N = 30 positive individuals treated. N = 30 positive individuals untreated. ** $P \leq 0.01$ and *** $P \leq 0.001$.

The prevalence of salmon gill poxvirus at the gills was 100 % within all the groups (except for the treated group; 93.3 %) (figure 3.20). There were no significant differences in density between the sampling groups (p-values are given in the appendix). The prevalence of the pathogens *Piscine orthoreovirus* 1 (PRV1) and *Paranucleospora theridion* (Nuc) at the gills was 100 % for all of the groups. The density of *Piscine orthoreovirus* 1 is significant higher for the group that died during treatment compared to the treated group ($P = 0.0011$) and the untreated group ($P = 0.0078$) (figure 3.21). The density for *P. theridion* is significant higher for the group that died during treatment compared to before treatment ($P < 0.0001$) and for the group that died during treatment compared to the treated group ($P < 0.0001$) and the untreated group ($P < 0.0001$) (figure *3*.22).

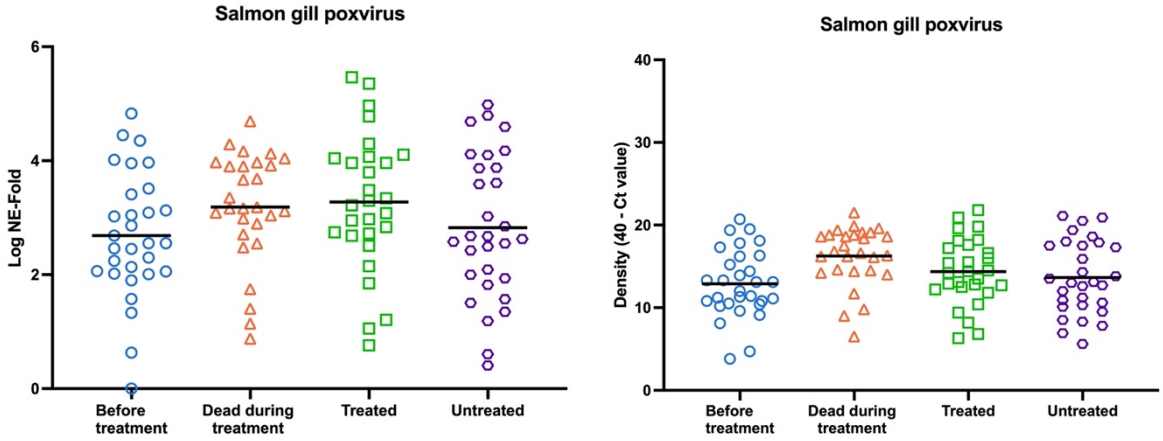

Figure 3.20 Density of Salmon gill poxvirus at the gills for all of the groups. Each point represent one individual fish. The data is presented as reversed Ct-values (40 – Ct-value) and Log NE-fold. The black line within each group represent the average Ct- value. N = 30 positive individuals before treatment. N = 28 postitive individuals died during treatement. N = 28 positive individuals treated. N = 30 positive individuals untreated.

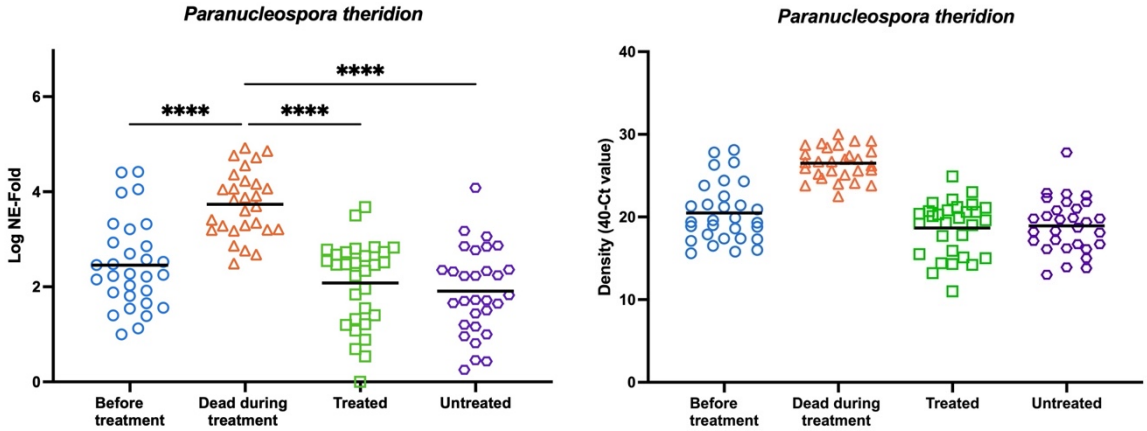

Figure 3.21 Density of *Paranucleospora theridion* at the gills for all of the groups. Each point represent one individual fish. The data is presented as reversed Ct-values (40 – Ct-value) and Log NE-fold. The black line within each group represent the average Ct- value. N = 30 postitive individuals for every group (except "dead during treatment" N = 28). **** $P \leq 0.0001$.

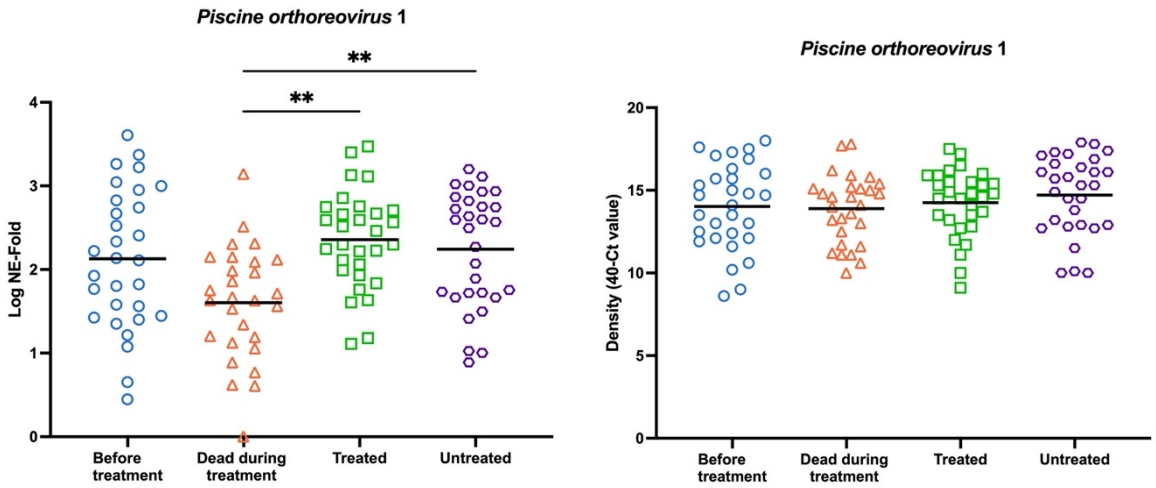

Figure 3.22 Density of *Piscine orthoreovirus* 1 at the gills for all of the groups. Each point represent one individual fish. The data is presented as reversed Ct-values (40 – Ct-value) and Log NE-fold. The black line within each group represent the average Ct- value. N = 30 positive individuals for every group (except "dead during treatment" N = 28). ** $P \leq 0.01$.

The prevalence of the pathogens *Piscine orthoreovirus* 1 (PRV1), Piscine myocarditis virus (PMCV) and *Paranucleospora theridion* (Nuc) in the analyzed kidney tissue within all of the four groups was close to 100 %. In addition, there were a low prevalence of the pathogens Infectious pancreatic necrosis virus (IPNV) and *Tetracapsuloides bryosalmonae* (PKX), with high Ct-values (appendix).

The diversity index for the gills sampled prior to treatment, the group the died during treatment, the treated group and the untreated group was 5.9, 5.9, 6.4 and 6.4 respectively. The diversity index for the kidney sampled prior to the treatment, the group the died during treatment, the treated group and the untreated group was 3.8, 3.8, 5,7, and 4.3 respectively.

The P-values for the remaining analyzed samples is given in the appendix.

3.2.3 Histology

The mean histological gill score for the different groups varied within some of the parameters for pathological changes, and for the other parameters the score was relatively similar. The pathological changes with highest score and biggest difference in score within the groups were parameter 1 (mucus cell hyperplasia), 5 (epithelial cell hyperplasia), 6 (thickening of distal primary lamellae) and 10 (inflammation) (figure 3.23). Most of the score given was 0, 1 and 2, but there were some pathological changes scored 3.

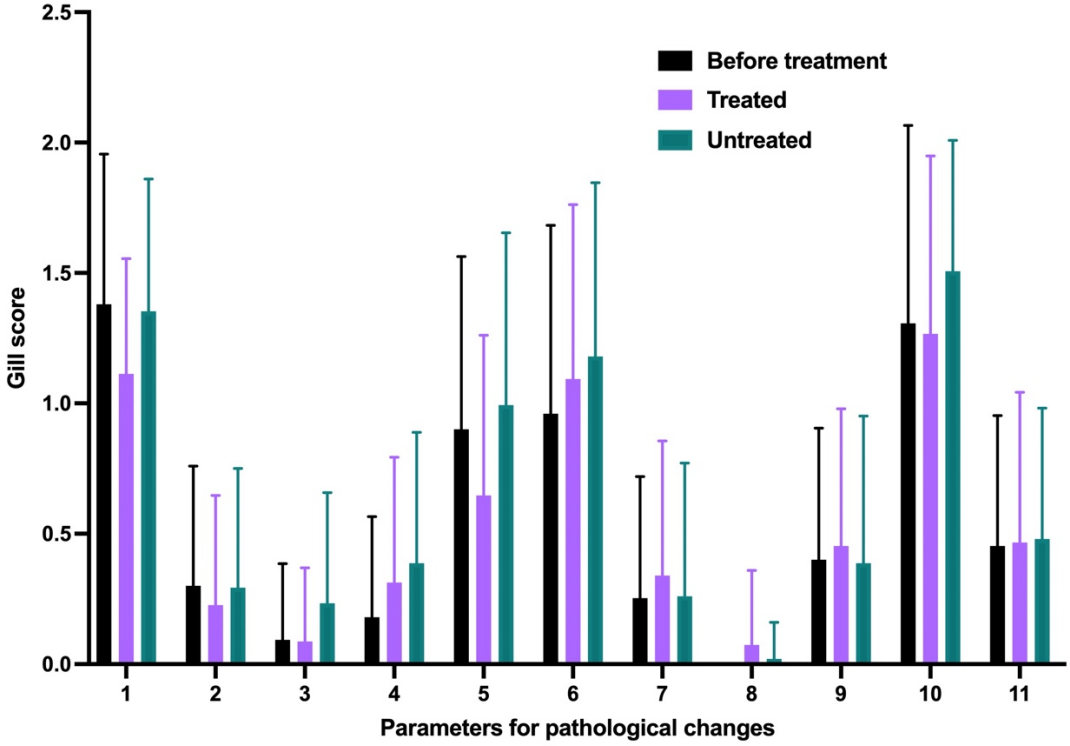

Figure 3.23 Mean histological gill score of the eleven pathological changes included in this study for before treatment, treated group and untreated group at locality LB. 1: Mucus cell hyperplasia. **2**: Clubbing. **3**: Lifting. **4**: Epithelial cell hypertrophy. **5**: Epithelial cell hyperplasia. **6**: Thickening of distal primary lamellae. **7**: Fresh aneurism. **8**: Bleeding aneurism. **9**: Old aneurism. **10**: Inflammation. **11**. Necrosis.

Most of the gills were clearly affected, with pathological changes compatible with AGD lesions which include segmental hyperplasia of the epithelial cells and caverns in addition to hypertrophy of the epithelial cells and inflammation (figure 3.24). Some of the gills were little affected, with few pathological changes (figure 3.25). Most of the lamellae were affected with hyperplasia of mucus cells, and early stages of lifting were observed (figure 3.26). In some of the histological sections, amoeba was observed, both free beside the gills or in caverns (figure 3.27 and 3.28). In addition, some of the sections had different types of aneurism, both fresh, bleeding and old aneurisms (figure 3.29). There was also observed different types of pathological changes compatible with epitheliocystis (figure 3.30).

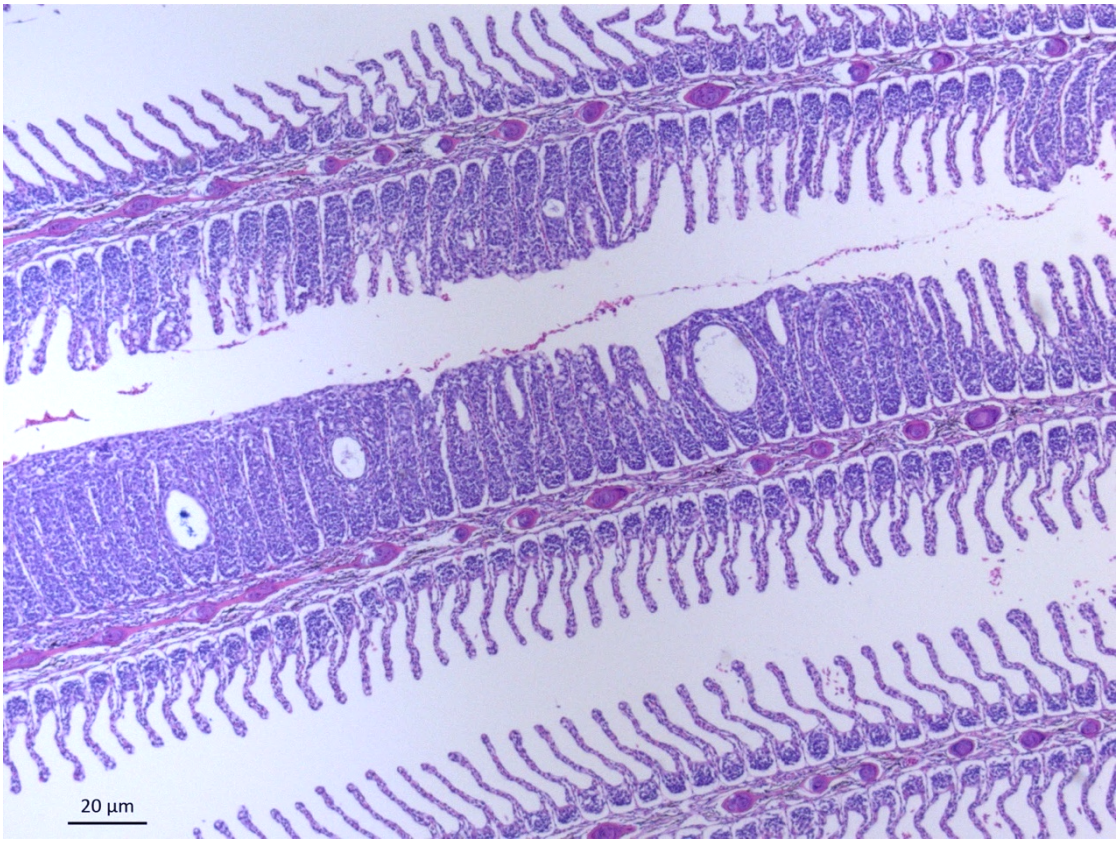

Figure 3.24 Histological section of fish nr. 105 (untreated fish) with pathological changes compatible with AGD lesions; segmental hyperplasia of the epithelial cells and caverns, hypertrophy of epithelial cells and inflammation. Scale: rod = 20 µm.

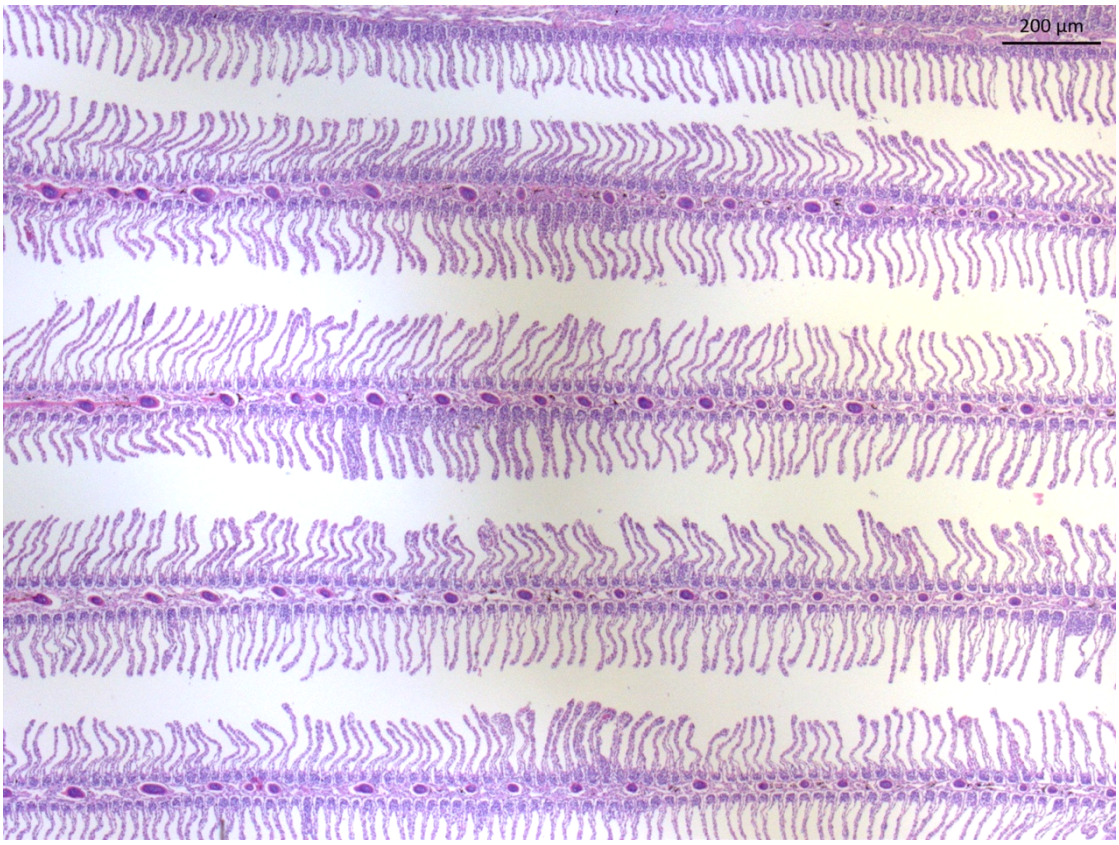

Figure 3.25 Histological section of fish nr. 65 (treated fish) with little pathological changes. Scale: rod = 200 µm.

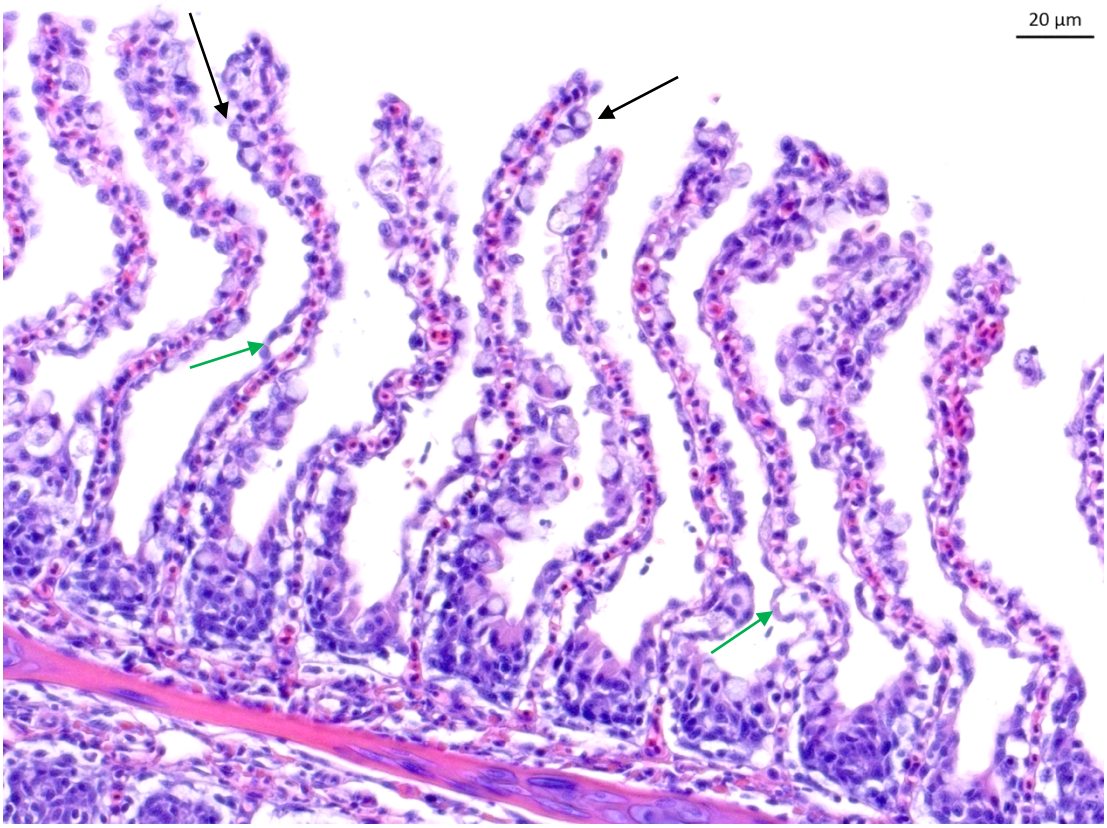

Figure 3.26 Histological section of fish nr. 102 (untreated fish) with pathological changes such as hyperplasia of mucus cells (black arrow) and early stages of lifting (green arrow). Scale: rod = 20 µm.

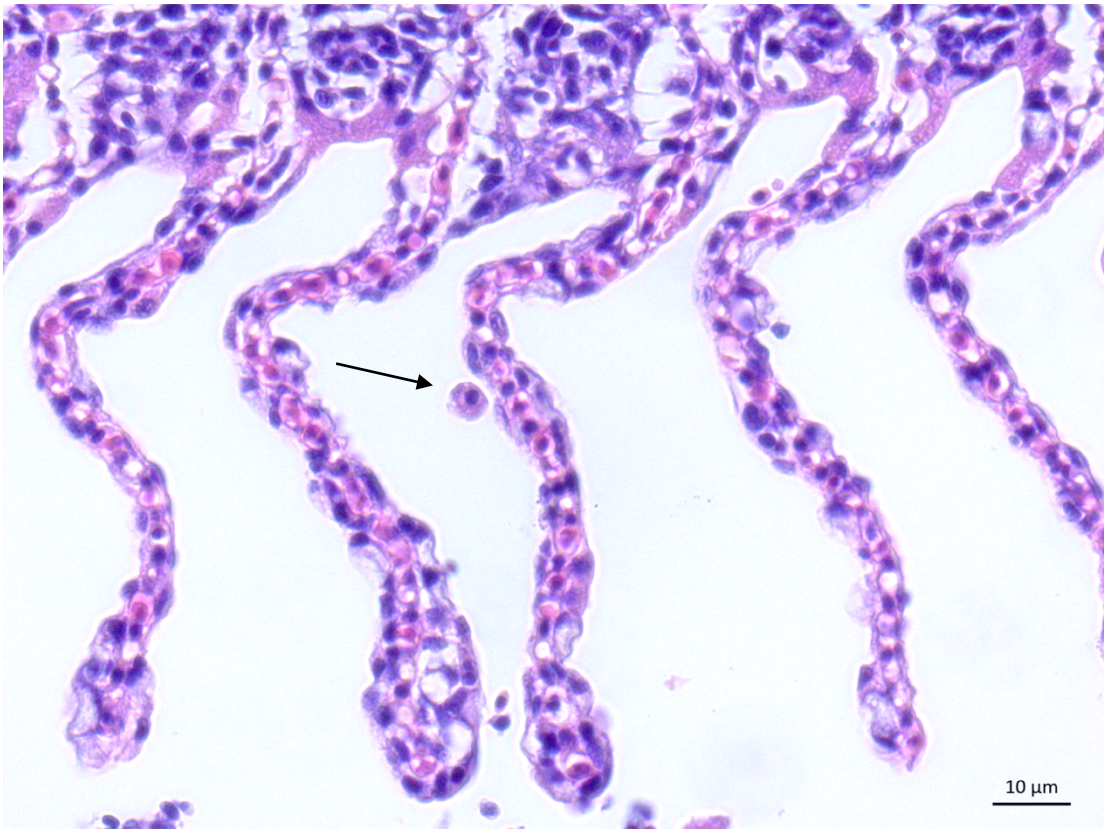

Figure 3.27 Histological section of fish nr. 65 (treated fish) with few pathological changes. Amoeba-like cell observed free beside the gills (black arrow). Scale: rod = 10 µm.

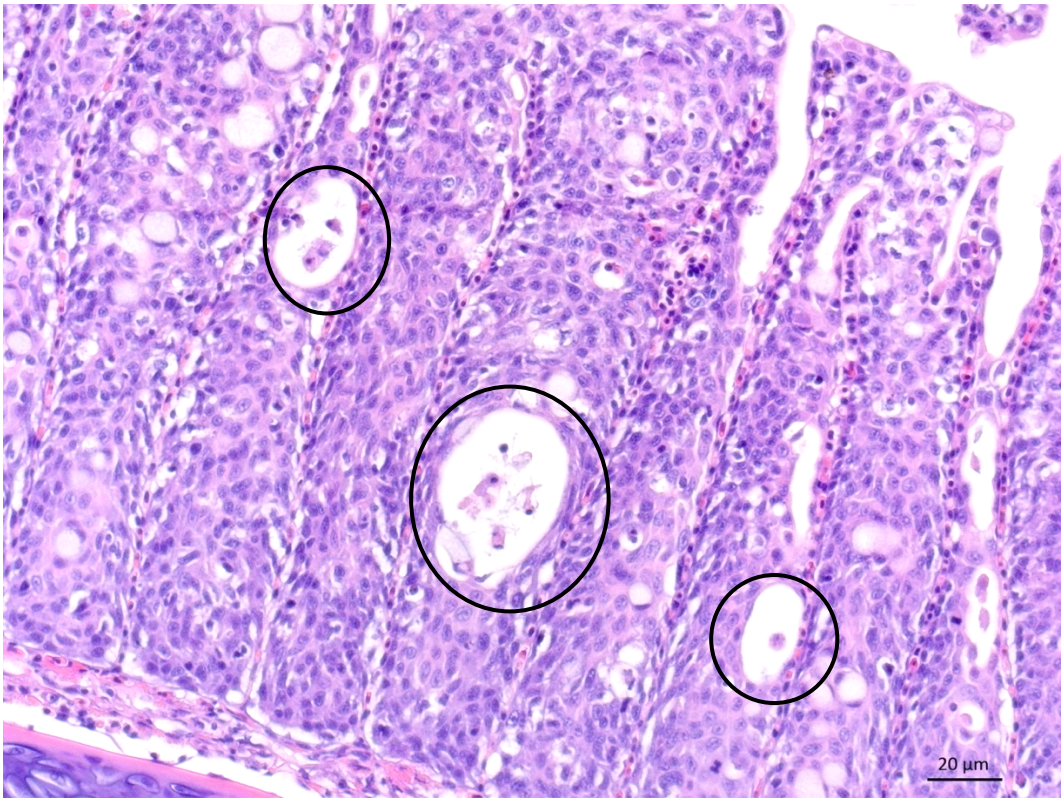

Figure 3.28 Histological section of fish nr. 20 (before treatment) with pathological changes compatible with AGD lesions; segmental hyperplasia of the epithelial cells and caverns, hypertrophy of epithelial cells and inflammation. Amoeba-like cells observed in the caverns (black circles). Scale: rod = 20 µm.

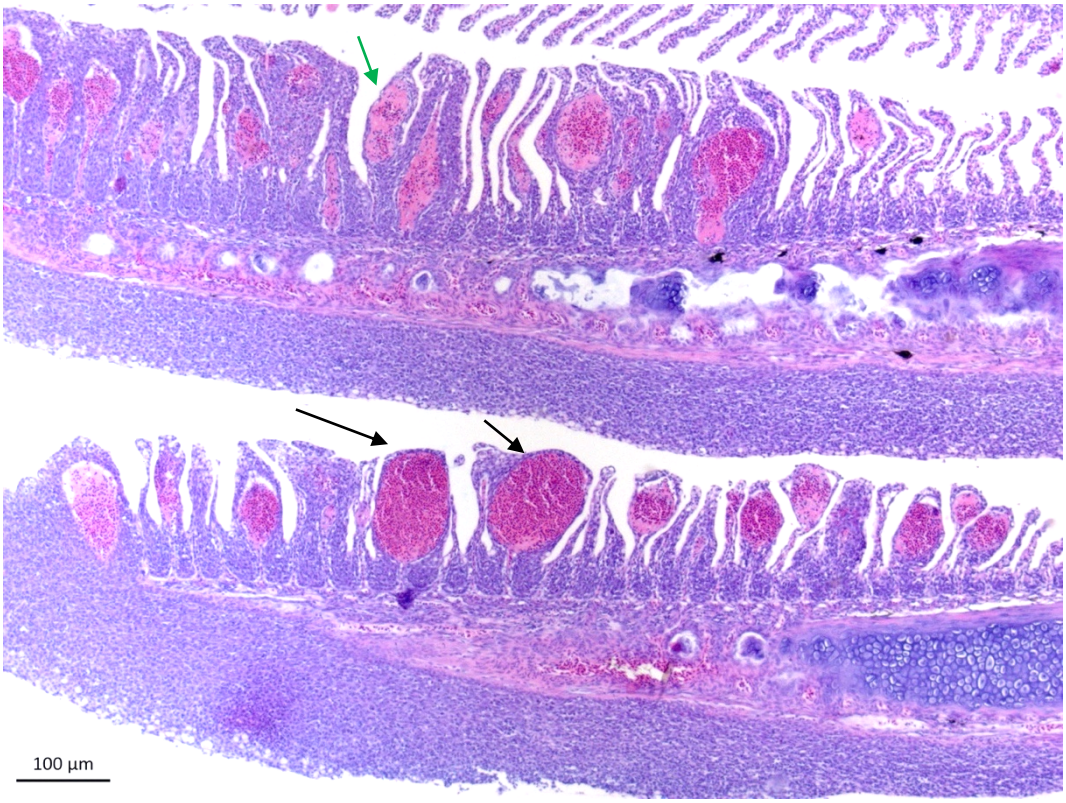

Figure 3.29 Histological section of fish nr. 103 (untreated fish) with clear pathological changes, such as fresh (black arrow) and old aneurisms (green arrow), in addition to thickening of distal primary lamellae. Scale: rod = 100 µm.

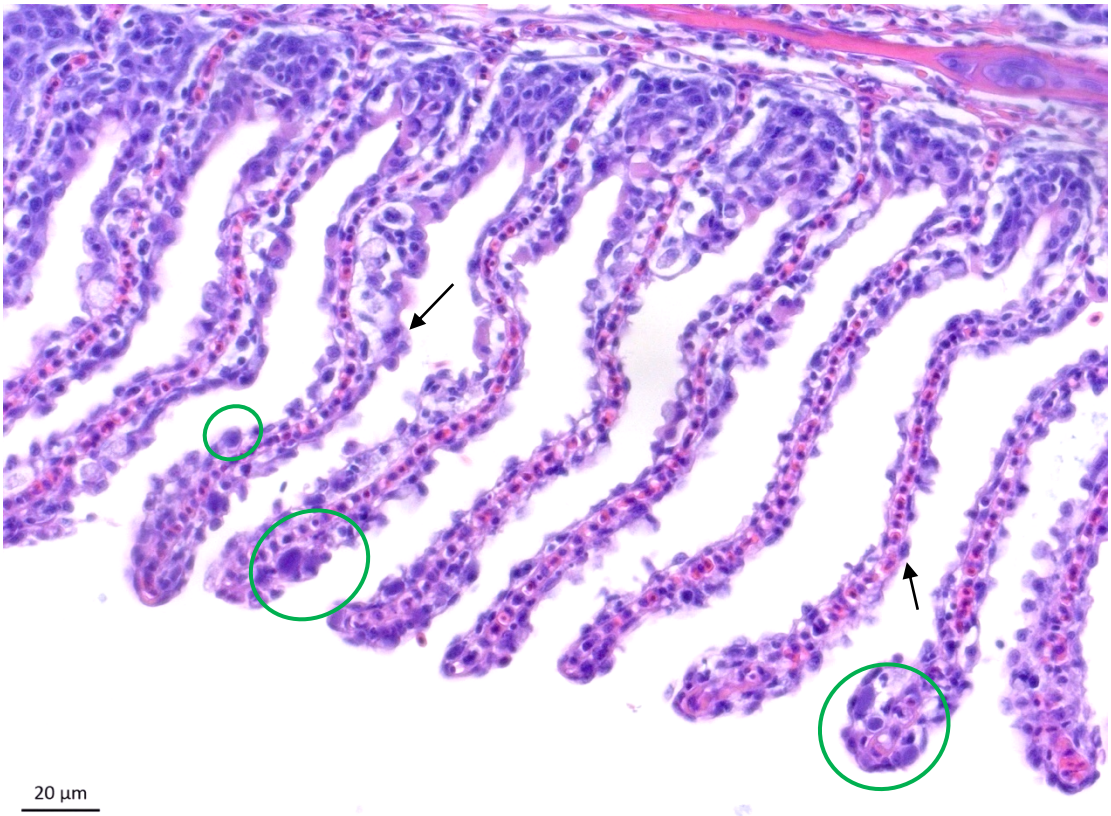

Figure 3.30 Histological section of fish nr. 102 (untreated fish) with hypertrophy of epithelial cells (black arrow) and epitheliocystis (red circle). Scale: rod = 20 µm.

4 Discussion

The present work represents the first study of the effect of freshwater treatment against AGD on the gill health of Atlantic salmon. Treatment and handling cause stressful situations which is a potential liability for the fish. More knowledge of how these stressful situations within the production affects the gill health of salmon and the associated microbiota is needed. Mapping the gill health and presence of pathogens before treatment revealed a considerable high load of diverse pathogens in the gills. The load of these pathogens changed after the treatment, and therefore provided valuable information of relevance for how stress (freshwater treatment) within production affects the gill health of the fish.

This thesis also represents a study of the possible effect microbiota may have on the virulence of clonal cultures of *P. perurans*. Currently, there are no published challenge experiments studying *P. perurans* and its associated microbiota. The relationship between microbiota and the phenotypic characteristics of *Paramoeba* is complex and not fully understood. Nonetheless there is a perception that the combination of *P. perurans* and specific bacteria is important for developing pathology associated with AGD. Nowak and Archibald (2018) have previously indicated that the bacteria are more than just a source of nutrition for the amoeba. Increased knowledge of how the microbiota affects *P. perurans*, and how infections of the amoeba are associated with microbial coinfections and dysbiosis on the gills is necessary. This challenge experiment indicated that the microbiota on the gills is an important factor for development of AGD. Therefore, new strategies for combating the disease can be used, and the welfare in fish farming improved.

Previous challenge experiments with *P. perurans* have been conducted by administrating primary isolates of the amoeba cultivated in a polyaxenic culture containing a diverse microbiota. More recent challenge studies have in contrary been performed using clones of *P. perurans*, still cultured within a media containing a high diversity of different bacteria (Bridle *et al.*, 2015; Dahle, 2015; Røed, 2016; Wiik-Nielsen *et al.*, 2016; Collins *et al.*, 2017; Kindt, 2017; Benedicenti, Pottinger, Collins, & Secombes, 2019a; Dahle *et al.*, 2020a). Administrating clones of a certain strain of *P. perurans* provides the advantage of describing the pathological changes associated with a specific clone. According to Benedicenti *et al.* (2019b), microbiota is suggested to constitute a factor that affects the growth and virulence of *P. perurans* as the composition of microbiota in the cultured clones are significant different. Still, there is a lack

of knowledge of the microbiota and how it possible affects the virulence of the amoeba and severity of AGD.

4.1 The importance of bacteria in the culture media of *P. perurans* clones for the development of AGD

Characterization of microbiota present in the culture media of *P. perurans* clones has revealed significant differences with regards to bacterial composition. *Vibrio splendidus* variants dominated bacteria isolations in the culture media of the high-virulent clone H20/16Pp and the low-virulent clone ST19/15Pp. *P. perurans* cultured in a pure culture of *V. splendidus* proved as sufficient growth as when grown in media containing microbiota from gills of salmon suffering from AGD. By transferring *P. perurans* clones to pure bacteria culture before infecting the salmon in the challenge, individual comparison of the virulence of the clones H20/16Pp and ST19/15Pp could be tested.

According to Zilberg, Gross, and Munday (2001); Morrison, Crosbie, and Nowak (2004); Collins *et al.* (2017) the correlation between challenge dose and the ability to develop gill lesions (AGD) is positive, where higher challenge doses provide higher gill score and more severe gill lesions. This implies the importance of adequate amount of amoeba in each tank. Thus, based on previous challenge experiments (Røed, 2016; Dahle *et al.*, 2020a), the challenge dose was set to 1000 amoeba/L.

Low numbers of lesions on the gills when scoring was observed, both during sampling at 27 dpi and 32 dpi. However, the tank with the highest mean gill score and highest number of gill surfaces with registered gill score was sampled from tank 4 infected with clone ST19/15Pp grown in normal media. Based on the results from a challenge conducted in 2016, the low number of observed lesions on the gills was not expected for the two clones used in the current study. Previous, the same high- and low-virulent clones have revealed significant differences in gill score and density in the gill tissue (Kindt, 2017). There were no significant differences in gill score at 27 dpi of fish challenged with the high-virulent clone cultured in normal media (H20/16Pp), in pure culture of *V. splendidus* (V.spl.H20) and normal media containing microbiota from the gills of farmed salmon during the first isolation of H20/16Pp. The fish challenged with the low-virulent clone cultured on normal media (ST19/15Pp) showed clear difference in the gill score compared to fish challenged with the clone cultured on pure culture

of *V. splendidus* (V.spl.ST19). The lack lesions on the gills appear to be somewhat related to the virulence of the clones. However, even if the virulence of the different clones are stable at genomic levels, the virulence might not be stable at the gene expression level (Collins *et al.*, 2017).

Røed (2016) and Dahle *et al.* (2020a) have carried out challenge experiments illustrating clear differences in the virulence for the different clones of *P. perurans*. Evidence indicates that the water temperature contributes to increased virulence of the clonal isolates of *P. perurans*, as well as increased ability for transmission, when the temperature rises from 12 to 16 °C (Kindt, 2017). The temperature used in this challenge experiment was 16 °C, which should be an optimal temperature for the virulence test. The elevated water temperature can constitute increased risk of severe clinical signs of AGD (Munday *et al.*, 2001), and increase the ability of adhesion of the amoeba at the gills *in vitro* (Benedicenti, Secombes, McCarthy, & Collins, 2016). Therefore, it is believed the temperature is not associated with the low gill scores observed in this study.

When the clones are passaged *in vitro*, loss of virulence in different pathogens is common, including the amoeba (Jellett & Scheibling, 1988; Ram, Khurana, Singh, & Khurana, 1992; Song, Santi, Evensen, & Vakharia, 2005; Veríssimo *et al.*, 2013; Songe, Thoen, Evensen, & Skaar, 2014). Likewise, the possibility for the isolates in this study to lose their virulence during clonal culture and passages may be present. Crosbie *et al.* (2012) believed they were the first to successfully culture the amoeba with continued virulence after 125 days and fulfilled Koch's postulate, and Bridle *et al.* (2015) proposed that the same isolate lost virulence after 3 years in clonal culture. The isolates used in this challenge experiment have been in clonal culture since 2015 (low-virulent clone) and 2016 (high-virulent clone), and the number of passages they have been through was 96 (H20/16Pp) and 103 (ST19/15Pp). As the inoculums contained high numbers of live amoebae there are no indications that the number of passages *in vitro* have resulted in a reduced viability of the amoebae. This corresponds to the fact that amoeba cultivated on continuous culture for more than 2.5 years that have remained their virulence (Kindt, 2017)

Even though the gills contained few lesions, the clones used in the challenge were still able to attach to the gills due to positive real time RT-PCR results. When the challenge was terminated, all salmon tested negative for the amoeba. Previous challenges using the same clones have

resulted in extreme density of the amoeba on the gill tissues and reduced mobility of the fish (Kindt, 2017). At 27 dpi, the real time RT-PCR results showed that all tanks, except tank 4, were negative for *P. perurans*. 13 of 17 individuals in tank 4 tested positive for *P. perurans* (Ct-value range: 18.4-31.9, average = 22.42). These results indicate that even though tanks 1-4 were challenged with 1000 amoeba/L, the high-virulent clone was unable to maintain infection on the gills. Between the challenge experiment conducted in December 2016 until October 2020, the H20/16Pp clone might have lost the ability to establish infections and inflict damage on the gills. The ST19/15Pp clone was able to infect the gills with the clone cultured in normal media, but for the clone cultured in pure culture of *V. splendidus* this ability was lost. Assuming the clones have not mutated to avirulent clones during culturing, the explanation for loss of virulence must be connected to the culture conditions and possible change in the microbiota in the culture media. This hypothesis is supported by the difference between the ST19/15Pp clone grown in the original media and grown in media containing *V. splendidus* only, seen in the present study.

4.2 Freshwater treatment against AGD

The locality was diagnosed with amoebic gill disease prior to treatment, and the real time RT-PCR results indicated high diversity of pathogens on the gills. Theses pathogens seemed to be of importance for the gill health. Although there was slightly increased mortality during the treatment, the mortality for the treated cage dropped after treatment. The identification of numerous pathogens on the gills corresponds with possible reduced immune response of the host as a result of recently performed treatments (Powell *et al.*, 2001; Rodger, 2014; Powell *et al.*, 2015; Oldham *et al.*, 2016; Hytterød *et al.*, 2017b). Due to already elevated mortality prior to the treatment, the mortality associated with the freshwater treatment was most likely a result of reduced gill health rather than the treatment itself.

This field experiment has shown that freshwater bathing is successful in reducing the number of amoebae on the gills of Atlantic salmon. The prevalence of *P. perurans* was reduced from 100 % prior to treatment to 33.3 % 8 days after treatment. MNE-values, showed that the density on the gills, have been significantly reduced for the treated group and the group that died during treatment. This observation is consistent with previous studies Wiik, Andersen, Uglenes, and Egidius (1989); Fast, Hosoya, Johnson, and Afonso (2008). The remaining amoeba after treatment is probably a result of occupation of the lacunae structure formed in the gills and

could represent a risk for reinfection if they survive Sveen, Øverland, Karlsbakk, and Nylund (2012); Herrero *et al.* (2018). Another possible explanation for the amoeba still being present in the gills could be horizontal transmission from the untreated cage. The mechanism of transmission is not fully understood and should be further investigated.

Previous studies have shown a correlation between pathology on the gills (measured by gill scoring and histopathology) and increased amount of *P. perurans* on the gill tissue and the surroundings (Kent *et al.*, 1988; Adams & Nowak, 2001). The salmon in all groups had severe gill pathology, before treatment, treated and untreated (figure 3.11 and figure 3.23). The freshwater bathing showed no distinct differences in the gill score and histopathological score between the groups. Although freshwater treatment against *P. perurans* have shown a reducing effect on the gill score (Morrison *et al.*, 2004; Dahle, 2015; Røed, 2016; Collins *et al.*, 2017; Haugland *et al.*, 2017), some gill arches in the present study still had a score of 2-3 after treatment. Wiik (2020) observed in a challenge experiment that it generally takes longer for the reduction of patches to be visible macroscopic. These findings are consistent with the high gill score registered after treatment in the current study.

4.2.1 Prevalence and density of microparasites

PCR screening prior to treatment is useful to detect early infections, and to assess efficacy of treatment (Marcos-López & Rodger, 2020). The salmon tested positive for more than 10 pathogens, some of them associated with gill disease (GD), when analyzing the gill tissue. The load of some pathogens was reduced after the freshwater treatment, including *P. perurans*, *Ichthyobodo* spp, *Cand.* S. salmonis and *Cand.* P. salmonis. Other pathogens had relatively similar density, or elevated density within the groups, including PRV1, *P. theridion*, SPGV, PMCV and *Cand.* B. cysticola.

Both density and prevalence of *P. perurans* were significantly reduced for the treated group, as expected due to previous studies (Parsons *et al.*, 2001; Powell *et al.*, 2015; Marcos-López & Rodger, 2020). The freshwater will inflict osmotic shock on the amoebae and reduce the mucus viscosity on the gills (Clark *et al.*, 2003; Adams & Nowak, 2004b; Roberts & Powell, 2008). Hence, the amoeba can more easily be removed from the gills during freshwater bathing and result in an effective treatment.

The prevalence of *Ichthyobodo* spp. was also significantly reduced within the treated group and the group that died during treatment (figure 3.15). The real time RT-PCR assay "Costia" can detect both *Ichthyobodo necator* and *Ichthyobodo salmonis*. While *I. necator* are only present in freshwater, *I. salmonis* can be present in both seawater and freshwater (Isaksen *et al.*, 2011; Isaksen *et al.*, 2012). Hence it is though that *I. salmonis* is the pathogen present on the gills. It may seem like *Ichthyobodo salmonis* needs more time to readjust during a freshwater treatment. It is suggested that *Ichthyobodo* spp. disappears from dead hosts and will die after 30-60 minutes while free-swimming (Isaksen, 2013). Also, the parasite is believed to survive on dead hosts for >30 hours or in sediments for several days (Isaksen, 2013). The result in this thesis indicates that the parasite most likely left the host that died during treatment. Studies of gill diseases have considered infections with *Ichthyobodo* spp. as secondary infection in relation to gill diseases (Draghi *et al.*, 2004; Young *et al.*, 2007; Nylund *et al.*, 2010; Steinum *et al.*, 2010; Isaksen *et al.*, 2011; Nylund *et al.*, 2011; Mitchell *et al.*, 2013; Nylund *et al.*, 2015; Blandford, Taylor-Brown, Schlacher, Nowak, & Polkinghorne, 2018). Hence, there is no clear indications that infections with *Ichthyobodo* spp. alone makes the fish less robust for treatment and facilitate mortality.

The prevalence of *Cand.* S. salmonis was significantly reduced after treatment. The bacteria have been isolated from fish with *P. perurans* and shown to be able to grow inside this amoeba. Therefore *Cand.* S. salmonis is believed to be an endosymbiont or a parasite of *P. perurans* (Nylund *et al.*, 2018a). Due to the associations between *Cand.* S. salmonis and *P. perurans*, the reduction of density of the bacteria was expected as the density of the amoeba was reduced. *Cand.* S. salmonis was also observed in salmon where the amoeba was absent, and vice versa. This is supported by findings of the bacteria not being an obligate symbiont for the amoeba (Nylund *et al.*, 2018b).

The pathogen PMCV was identified with 100 % prevalence in all groups, both in the gill- and kidney tissue. PMCV is the causative agent for cardiomyopathy syndrome (CMS) (Haugland *et al.*, 2011; Garseth, Fritsvold, Svendsen, Bang Jensen, & Mikalsen, 2018). The virus causes inflammation in the atrium, spongious ventricle, compact ventricle and bulbus arteriosus of the heart, which could lead to heart rupture during stress (Nylund *et al.*, 2011; Gunnarsson *et al.*, 2017b). It is believed this may have happened in this study, due to significant higher density and low average Ct-values of PMCV in the group that died during treatment. Both kidney- and gill tissue had low Ct-values, which correlates to previous studies showing that increased viral

load over time may cause systemic infection (Kvellestad *et al.*, 2005; Rodger, Murphy, Mitchell, & Henry, 2011; Mitchell *et al.*, 2013). Hence, it is thought that PMCV is of importance for the salmon health in relation to the observed mortality during treatment.

No distinct differences in density were registered for SPGV. The prevalence of SGPV was almost 100 % for all groups, and the highest density in gill tissue was registered for the group that died during treatment. This result is not relatable to a study indicating the level of virus is reduced in the tissue sampled from dead hosts (Gjessing *et al.*, 2015). Gjessing, Thoen, Tengs, Skotheim, and Dale (2017) suggest the possibility for SGPV as a primary pathogen in gill disease exacerbating secondary pathogens and the consequences of those. Thus, SGPV is of importance for the results in this study, and the possibility for the virus to facilitate mortality during treatment needs to be taken into consideration. SGPV is strongly associated with gill diseases, and studies have shown that the virus combined with AGD could lead to high mortality (Gjessing *et al.*, 2015; Thoen *et al.*, 2020). This corresponds to the thought that the virus disturbs the epithelial barriers on the gills, possibly compromising the innate immunity towards other agents (Gjessing *et al.*, 2017). Thus, it is believed that SGPV may have a role in the observed mortality at the study site.

The prevalence of *Cand.* B. cysticola was 100 % in the gills of all groups, with the lowest Ct-values within the group that died during treatment. Tolås (2012) have confirmed the presence of *Cand.* B. cysticola in kidney tissues, indicating that *Cand.* B. cysticola may occur systemic. In this study, the density of *Cand.* B. cysticola was higher in the gill tissue than in the kidney tissue, confirming the gills as the primary target organs. Presence of *Cand.* B. cysticola in kidneys could be a result of leakage from the gill tissues into the blood stream. Several studies indicate that *Cand.* B. cysticola is a primary pathogen for epitheliocystis (Ferguson, Poppe, & Speare, 1990; Garseth *et al.*, 2018; Frisk *et al.*, 2020), and associated with histological changes causing respiratory problem (Haugland *et al.*, 2011; Timmerhaus *et al.*, 2011). Epitheliocystis was observed at high levels in the histopathological sections, therefore it is believed that *Cand.* B. cysticola could have contributed to reduced gill health and mortality during the freshwater treatment.

All the fish in this study tested positive for PRV1 shown to cause the viral disease heart- and skeletal muscle inflammation (HSMI) in Atlantic salmon. Handling of the fish during treatments can influence the outcome for the disease (Gjessing *et al.*, 2015; Thoen *et al.*, 2020).

PRV1 was present in both gill- and kidney tissue, and the density of PRV1 was higher in the kidney. This corresponds to the fact that the virus infects erythrocytes which could circulate from kidney to gills Gjessing *et al.* (2015). Significant higher density of PRV1 for the group that died during treatment reveals that the virus may play for the gill health during treatments. Although the accumulated mortality due to HSMI usually ranges from 0-20 % (Wessel *et al.*, 2017; Dhamotharan *et al.*, 2020), the virus's effect on the heart may weaken the fish prior to treatment. Therefore, PRV1 as a factor affecting the health of salmon negatively needs to be considered in relation to the mortality during treatment.

The prevalence of *P. theridion* was 100 % in all groups, in both gill- and kidney tissue. According to previous findings by Nylund *et al.* (2010), the microsporidium has relative similar density in organs that are positive. This is compatible with the density of *P. theridion* in this study, as the density was relatively similar in the gills and kidney. How this microsporidium affects the gills alone is not well understood, but it is associated with other histopathological changes in the gills, such as hyperplasia, hypertropia and inflammation (Toenshoff *et al.*, 2012; Mitchell *et al.*, 2013). These changes were observed in the histopathological sections studied, indicating that the parasites involvement in the gill pathology.

4.2.2 Histopathology and gill score in relation to density

The histopathology study showed clear pathology on the gills, believed to be associated with the high load of pathogens present. Even though there was a slight reduction of the gill score after treatment among some of the parameters (figure 3.23), there was no significant histopathological changes between the observed groups.

The histological sections showed pathological changes compatible with AGD and the real time RT-PCR identified significant amounts of *P. perurans*, but few amoebae were observed in the sections. Most of the amoeba observed in the sections were mostly found within the lacunae formed by AGD lesions, indicating that this formation can in fact protect the amoeba from the treatment. It is possible that the processing for histology may have washed away the amoeba (Nylund, pers. com.). The pathogen that could be observed in the sections were epitheliocystis, and a few amoebae.

There was a higher level of old aneurisms present in the gills compared to fresh aneurism and bleeding aneurism. This indicates that damages in the gill tissue were present prior to the

treatment, which is expected as the fish were diagnosed with gill disease. The level of fresh and old aneurism was somehow higher for the treated fish than for the untreated fish, both prior and post treatment. It is not known whether this is a result of the treatment or a random result (sampling error).

5 Conclusion

The challenge experiment showed that although the high- and low virulent clones of *P. perurans* grew well in pure culture of *V. splendidus* and in the media containing microbiota from gills of salmon suffering for AGD, the clones where not able to establish a lasting infection on the gills. The high virulent clone (H20/16Pp) have most likely lost the ability to infect salmon and inflict damages on the gills. The low-virulent clone (ST19/15Pp) grown in original media was still able to establish infection on the gills, but the clone grown in pure *V. splendidus* could not infect salmon and inflict damages on the gills. After this challenge, it is believed that the clone's loss of ability to induce AGD in salmon is related to the change in microbiota in the culture.

The field experiment showed no significant histopathological changes between the groups. Hence, this indicates that the freshwater treatment had little effect on the histopathological changes. The load of some pathogens was reduced after the freshwater treatment, including *P. perurans*, *Ichthyobodo* spp, *Candidatus* Syngnamydia salmonis and *Candidatus* Piscichlamydia salmonis. Other pathogens had relative similar density in all groups, including *Piscine orthoreovirus* 1, *Paranucleospora theridion* and Salmon gill poxvirus, and some pathogens (Piscine myocarditis virus and *Candidatus* Branchiomonas cisticola) had a higher density, especially in the fish that died during treatment. There is little knowledge on how the different pathogens affects the outcome of treatments alone. All the identified pathogens together will most likely reduce the gill health of the fish and affect the outcome of a treatment. It is believed that the sum of these pathogens, especially the viruses, and the status of gill health prior to the treatment is related to the mortality during treatment rather than the treatment itself. Due to the fact that there was no increased mortality after the freshwater treatment, the method did not have any negative effect on the gill health.

5.1 Future perspective

During the challenge experiment, the high-virulent and low-virulent clone cultured in pure culture of *V. splendidus* did not inflict infection and damage on the gills. Due to a large variation of bacteria species and significant differences in the composition of the cultures, further studies on how other bacteria species present in the culture may affect the virulence of the *P. perurans* clones needs to be investigated. If the microbiota on the gills is of importance for developing

AGD, this could lead to new strategies in controlling and prevention of the disease, and thus improve the welfare in commercial production of Atlantic salmon.

The field study indicated that the sum of pathogens on the gills prior to treatment is related to the mortality. Further studies should consider how different pathogens on the gills alone in relation to *P. perurans* will affect the gill health and outcome of treatment. In this study, the fish sampled in the field were tracked for 8 days post treatment. Therefore, the change in pathology on the gills and gill score after two weeks post treatment should be further assessed.

6 References

Adams, M. B., Crosbie, P. B., & Nowak, B. F. (2012). Preliminary success using hydrogen peroxide to treat Atlantic salmon, *Salmo salar* L., affected with experimentally induced amoebic gill disease (AGD). *J Fish Dis, 35*(11), 839-848. doi:10.1111/j.1365-2761.2012.01422.x

Adams, M. B., Ellard, K., & Nowak, B. F. (2004). Gross pathology and its relationship with histopathology of amoebic gill disease (AGD) in farmed Atlantic salmon, *Salmo salar* L. *J Fish Dis, 27*(3), 151-161. doi:10.1111/j.1365-2761.2004.00526.x

Adams, M. B., & Nowak, B. F. (2001). Distribution and structure of lesions in the gills of Atlantic salmon, *Salmo salar* L., affected with amoebic gill disease. *Journal of Fish Diseases, 24*(9), 535-542. doi:https://doi.org/10.1046/j.1365-2761.2001.00330.x

Adams, M. B., & Nowak, B. F. (2003). Amoebic gill disease: sequential pathology in cultured Atlantic salmon, *Salmo salar* L. *Journal of Fish Diseases, 26*(10), 601-614. doi:https://doi.org/10.1046/j.1365-2761.2003.00496.x

Adams, M. B., & Nowak, B. F. (2004a). Experimental amoebic gill disease of Atlantic salmon, *Salmo salar* L.: further evidence for the primary pathogenic role of *Neoparamoeba* sp. (Page, 1987). *J Fish Dis, 27*(2), 105-113. doi:10.1111/j.1365-2761.2004.00522.x

Adams, M. B., & Nowak, B. F. (2004b). Sequential pathology after initial freshwater bath treatment for amoebic gill disease in cultured Atlantic salmon, *Salmo salar* L. *J Fish Dis, 27*(3), 163-173. doi:10.1111/j.1365-2761.2004.00531.x

Al-Harbi, A. H., & Uddin, M. N. (2007). Seasonal trends in gill bacterial flora of hybrid tilapia, *Oreochromis niloticus*× *O. aureus*. *Journal of Applied Aquaculture, 19*(1), 61-70.

Andersen, L., Hodneland, K., & Nylund, A. (2010). No influence of oxygen levels on pathogenesis and virus shedding in Salmonid alphavirus (SAV)-challenged Atlantic salmon (*Salmo salar* L.). *Virology Journal, 7*(1), 198. doi:10.1186/1743-422X-7-198

Benedicenti, O., Collins, C., Wang, T., McCarthy, U., & Secombes, C. J. (2015). Which Th pathway is involved during late stage amoebic gill disease? *Fish & Shellfish Immunology, 46*(2), 417-425. doi:https://doi.org/10.1016/j.fsi.2015.07.002

Benedicenti, O., Pottinger, T. G., Collins, C., & Secombes, C. J. (2019a). Effects of temperature on amoebic gill disease development: Does it play a role? *J Fish Dis, 42*(9), 1241-1258. doi:10.1111/jfd.13047

Benedicenti, O., Secombes, C., McCarthy, U., & Collins, C. (2016). *Atlantic salmon physiological and immune response to amoebic gill disease and insight into the biology of the amoeba* (Vol. 53).

Benedicenti, O., Secombes, C. J., & Collins, C. (2019b). Effects of temperature on *Paramoeba perurans* growth in culture and the associated microbial community. *Parasitology, 146*(4), 533-542. doi:http://dx.doi.org/10.1017/S0031182018001798

Bermingham, M. L., & Mulcahy, M. F. (2004). Environmental risk factors associated with amoebic gill disease in cultured salmon, *Salmo salar* L., smolts in Ireland. *Journal of Fish Diseases, 27*(10), 555-571. doi:https://doi.org/10.1111/j.1365-2761.2004.00574.x

Bermingham, M. L., & Mulcahy, M. F. (2007). *Neoparamoeba* sp. and other protozoans on the gills of Atlantic salmon *Salmo salar* smolts in seawater. In (pp. 231-240). Amelinghausen, Federal Republic of Germany :.

Blandford, M. I., Taylor-Brown, A., Schlacher, T. A., Nowak, B., & Polkinghorne, A. (2018). Epitheliocystis in fish: An emerging aquaculture disease with a global impact. *Transbound Emerg Dis, 65*(6), 1436-1446. doi:10.1111/tbed.12908

Bloecher, N., Powell, M., Hytterød, S., Gjessing, M., Wiik-Nielsen, J., Mohammad, S. N., . . . Gjevre, A.-G. (2018). Effects of cnidarian biofouling on salmon gill health and development of amoebic gill disease. *PLOS ONE, 13*(7), e0199842. doi:10.1371/journal.pone.0199842

Boerlage, A. S., Ashby, A., Herrero, A., Reeves, A., Gunn, G. J., & Rodger, H. D. (2020). Epidemiology of marine gill diseases in Atlantic salmon (*Salmo salar*) aquaculture: a review. *Reviews in Aquaculture, 12*(4), 2140-2159. doi:https://doi.org/10.1111/raq.12426

Bridle, A. R., Crosbie, P. B. B., Cadoret, K., & Nowak, B. F. (2010). Rapid detection and quantification of *Neoparamoeba perurans* in the marine environment. *Aquaculture, 309*(1), 56-61. doi:https://doi.org/10.1016/j.aquaculture.2010.09.018

Bridle, A. R., Davenport, D. L., Crosbie, P. B., Polinski, M., & Nowak, B. F. (2015). *Neoparamoeba perurans* loses virulence during clonal culture. *Int J Parasitol, 45*(9-10), 575-578. doi:10.1016/j.ijpara.2015.04.005

Bridle, A. R., Morrison, R. N., Cupit Cunningham, P. M., & Nowak, B. F. (2006). Quantitation of immune response gene expression and cellular localisation of interleukin-1β mRNA in Atlantic salmon, *Salmo salar* L., affected by amoebic gill disease (AGD). *Veterinary Immunology and Immunopathology, 114*(1), 121-134. doi:https://doi.org/10.1016/j.vetimm.2006.08.002

Bruno, D. W., & Raynard, R. S. (1994). Studies on the use of hydrogen peroxide as a method for the control of sea lice on Atlantic salmon. *Aquaculture International, 2*(1), 10-18. doi:10.1007/BF00118529

Bustos, P. A., Young, N. D., Rozas, M. A., Bohle, H. M., Ildefonso, R. S., Morrison, R. N., & Nowak, B. F. (2011). Amoebic gill disease (AGD) in Atlantic salmon (*Salmo salar*) farmed in Chile. *Aquaculture, 310*(3), 281-288. doi:https://doi.org/10.1016/j.aquaculture.2010.11.001

Cahill, M. M. (1990). Bacterial flora of fishes: a review. *Microbial ecology, 19*(1), 21-41.

Clark, A., & Nowak, B. F. (1999). Field investigations of amoebic gill disease in Atlantic salmon, *Salmo salar* L., in Tasmania. *Journal of Fish Diseases, 22*(6), 433-443. doi:https://doi.org/10.1046/j.1365-2761.1999.00175.x

Clark, G., Powell, M., & Nowak, B. (2003). Effects of commercial freshwater bathing on reinfection of Atlantic salmon, *Salmo salar*, with Amoebic Gill Disease. *Aquaculture, 219*(1), 135-142. doi:https://doi.org/10.1016/S0044-8486(03)00020-6

Collins, C., Hall, M., Bruno, D., Sokolowska, J., Duncan, L., Yuecel, R., . . . MacKay, Z. (2017). Generation of *Paramoeba perurans* clonal cultures using flow cytometry and confirmation of virulence. *Journal of Fish Diseases, 40*(3), 351-365. doi:https://doi.org/10.1111/jfd.12517

Collins, C., Hall, M., Fordyce, M. J., & White, P. (2019). Survival and Growth in vitro of *Paramoeba perurans* Populations Cultured Under Different Salinities and Temperatures. *Protist, 170*(2), 153-167. doi:10.1016/j.protis.2018.11.003

Crosbie, P., Nowak, B., & Carson, J. (2003). Isolation of *Neoparamoeba pemaquidensis* Page, 1987 from marine and estuarine sediments in Tasmania. *BULLETIN-EUROPEAN ASSOCIATION OF FISH PATHOLOGISTS, 23*(5), 241-244.

Crosbie, P. B. B., Bridle, A. R., Cadoret, K., & Nowak, B. F. (2012). In vitro cultured *Neoparamoeba perurans* causes amoebic gill disease in Atlantic salmon and fulfils Koch's postulates. *International Journal for Parasitology, 42*(5), 511-515. doi:https://doi.org/10.1016/j.ijpara.2012.04.002

Crosbie, P. B. B., Bridle, A. R., Leef, M. J., & Nowak, B. F. (2010). Effects of different batches of *Neoparamoeba perurans* and fish stocking densities on the severity of amoebic gill disease in experimental infection of Atlantic salmon, *Salmo salar* L. *Aquaculture Research, 41*(10), e505-e516. doi:https://doi.org/10.1111/j.1365-2109.2010.02522.x

Dahle, O. M. V. (2015). *Experimental infections with Paramoeba perurans and AGD development in Atlantic salmon (Salmo salar) and ballan wrasse (Labrus bergylta). Are there host and P. perurans strain-related differences in infectivity and virulence?* University of Bergen,

Dahle, O. M. V., Blindheim, S. H., Nylund, A., Karlsbakk, E., Breck, O., Glosvik, H., & Andersen, L. (2020a). Atlantic salmon *Salmo salar* and ballan wrasse *Labrus bergylta* display different susceptibility to clonal strains of *Paramoeba perurans*. *Dis Aquat Organ, 140*, 55-72. doi:10.3354/dao03483

Dahle, S. W., Bakke, I., Birkeland, M., Nordøy, K., Dalum, A. S., & Attramadal, K. J. K. (2020b). Production of lumpfish (*Cyclopterus lumpus* L.) in RAS with distinct water treatments: Effects on fish survival, growth, gill health and microbial communities in rearing water and biofilm. *Aquaculture, 522*, 735097. doi:https://doi.org/10.1016/j.aquaculture.2020.735097

Dhamotharan, K., Bjørgen, H., Malik, M. S., Nyman, I. B., Markussen, T., Dahle, M. K., . . . Rimstad, E. (2020). Dissemination of *Piscine orthoreovirus*-1 (PRV-1) in Atlantic Salmon (*Salmo salar*) during the Early and Regenerating Phases of Infection. *Pathogens, 9*(2). doi:10.3390/pathogens9020143

Douglas-Helders, G. M., Weir, I. J., O'Brien, D. P., Carson, J., & Nowak, B. F. (2004). Effects of husbandry on prevalence of amoebic gill disease and performance of reared Atlantic salmon (*Salmo salar* L.). *Aquaculture, 241*(1), 21-30. doi:https://doi.org/10.1016/j.aquaculture.2004.07.026

Douglas-Helders, M., Nowak, B., Zilberg, D., & Carson, J. (2000). Survival of *Paramoeba pemaquidensis* on dead salmon: implications for management of cage hygiene. *Bulletin of the European Association of Fish Pathologists, 20*(3), 167-169.

Douglas-Helders, M., Saksida, S., Raverty, S., & Nowak, B. F. (2001). Temperature as a risk factor for outbreaks of Amoebic Gill Disease in farmed Atlantic salmon (*Salmo salar*). *Bulletin- European Association of Fish Pathologists, 21*, 114-116.

Downes, J., Henshilwood, K., Collins, E., A, R., O'Connor, I., Rodger, H., . . . 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*. doi:10.3354/aei00150

Downes, J. K., Yatabe, T., Marcos-Lopez, M., Rodger, H. D., MacCarthy, E., O'Connor, I., . . . Ruane, N. M. (2018). Investigation of co-infections with pathogens associated with gill disease in Atlantic salmon during an amoebic gill disease outbreak. *Journal of Fish Diseases, 41*(8), 1217-1227. doi:https://doi.org/10.1111/jfd.12814

Draghi, A., 2nd, Popov, V. L., Kahl, M. M., Stanton, J. B., Brown, C. C., Tsongalis, G. J., . . . Frasca, S., Jr. (2004). Characterization of "*Candidatus* piscichlamydia salmonis" (order Chlamydiales), a chlamydia-like bacterium associated with epitheliocystis in farmed Atlantic salmon (*Salmo salar*). *J Clin Microbiol, 42*(11), 5286-5297. doi:10.1128/jcm.42.11.5286-5297.2004

Dyková, I., Fiala, I., Lom, J., & Lukeš, J. (2003). *Perkinsiella* amoebae-like endosymbionts of *Neoparamoeba* spp., relatives of the kinetoplastid *Ichthyobodo*. *European Journal of Protistology, 39*(1), 37-52. doi:https://doi.org/10.1078/0932-4739-00901

Dyková, I., Figueras, A., & Peric, Z. (2000). *Neoparamoeba* Page, 1987: light and electron microscopic observations on six strains of different origin. *Dis Aquat Organ, 43*(3), 217-223. doi:10.3354/dao043217

Dyková, I., Nowak, B., Pecková, H., Fiala, I., Crosbie, P., & Dvoráková, H. (2007). Phylogeny of *Neoparamoeba* strains isolated from marine fish and invertebrates as inferred from SSU rDNA sequences. In (pp. 57-65). Amelinghausen, Federal Republic of Germany :.

Dyková, I., Nowak, B. F., Crosbie, P. B., Fiala, I., Pecková, H., Adams, M. B., . . . Dvoráková, H. (2005). *Neoparamoeba branchiphila* n. sp., and related species of the genus *Neoparamoeba* Page, 1987: morphological and molecular characterization of selected strains. *J Fish Dis, 28*(1), 49-64. doi:10.1111/j.1365-2761.2004.00600.x

Egan, S., & Gardiner, M. (2016). Microbial Dysbiosis: Rethinking Disease in Marine Ecosystems. *Frontiers in Microbiology, 7*(991). doi:10.3389/fmicb.2016.00991

English, C. J., Tyml, T., Botwright, N. A., Barnes, A. C., Wynne, J. W., Lima, P. C., & Cook, M. T. (2019). A diversity of amoebae colonise the gills of farmed Atlantic salmon (*Salmo salar*) with amoebic gill disease (AGD). *Eur J Protistol, 67*, 27-45. doi:10.1016/j.ejop.2018.10.003

Fast, M. D., Hosoya, S., Johnson, S. C., & Afonso, L. O. (2008). Cortisol response and immune-related effects of Atlantic salmon (*Salmo salar* Linnaeus) subjected to short- and long-term stress. *Fish Shellfish Immunol, 24*(2), 194-204. doi:10.1016/j.fsi.2007.10.009

Feehan, C., Johnson-MacKinnon, J., Scheibling, R., Lauzon-Guay, J.-S., & Simpson, A. (2013). Validating the identity of *Paramoeba* invadens, the causative agent of recurrent mass mortality of sea urchins in Nova Scotia, Canada. *Diseases of Aquatic Organisms, 103*, 209-227. doi:10.3354/dao02577

Ferguson, H. W., Poppe, T., & Speare, D. J. (1990). Cardiomyopathy in farmed Norwegian salmon. In (pp. 225-231). Amelinghausen, Federal Republic of Germany :.

Fisk, D., Powell, M., & Nowak, B. F. (2002). The Effect of Amoebic Gill Disease and Hypoxia on Survival and Metabolic Rate of Atlantic Salmon (*Salmo salar*). *Bulletin of the European Association of Fish Pathologists, 22*.

Fringuelli, E., Gordon, A. W., Rodger, H., Welsh, M. D., & Graham, D. A. (2012). Detection of *Neoparamoeba perurans* by duplex quantitative Taqman real-time PCR in formalin-fixed, paraffin-embedded Atlantic salmonid gill tissues. *Journal of Fish Diseases, 35*(10), 711-724. doi:https://doi.org/10.1111/j.1365-2761.2012.01395.x

Frisch, K., Småge, S. B., Johansen, R., Duesund, H., Brevik, Ø. J., & Nylund, A. (2018). Pathology of experimentally induced mouthrot caused by *Tenacibaculum maritimum* in Atlantic salmon smolts. *PLOS ONE, 13*(11), e0206951. doi:10.1371/journal.pone.0206951

Frisk, M., Høyland, M., Zhang, L., Vindas, M. A., Øverli, Ø., & Johansen, I. B. (2020). Intensive smolt production is associated with deviating cardiac morphology in Atlantic salmon (*Salmo salar* L.). *Aquaculture, 529*, 735615. doi:https://doi.org/10.1016/j.aquaculture.2020.735615

Gamble, M., & Bancroft, J. D. (2008). *Theory and practice of histological techniques* (6th ed. ed.). Philadelphia: Churchill Livingstone Elsevier.

Garseth, Å. H., Fritsvold, C., Svendsen, J. C., Bang Jensen, B., & Mikalsen, A. B. (2018). Cardiomyopathy syndrome in Atlantic salmon *Salmo salar* L.: A review of the current state of knowledge. *Journal of Fish Diseases, 41*(1), 11-26. doi:https://doi.org/10.1111/jfd.12735

Gjessing, M. C., Thoen, E., Tengs, T., Skotheim, S. A., & Dale, O. B. (2017). Salmon gill poxvirus, a recently characterized infectious agent of multifactorial gill disease in freshwater- and seawater-reared Atlantic salmon. *J Fish Dis, 40*(10), 1253-1265. doi:10.1111/jfd.12608

Gjessing, M. C., Yutin, N., Tengs, T., Senkevich, T., Koonin, E., Rønning, H. P., . . . Dale, O. B. (2015). Salmon Gill Poxvirus, the Deepest Representative of the Chordopoxvirinae. *Journal of virology, 89*(18), 9348-9367. doi:10.1128/jvi.01174-15

Grave, K., Horsberg, T. E., Lunestad, B. T., & Litleskare, I. (2004). Consumption of drugs for sea lice infestations in Norwegian fish farms: methods for assessment of treatment patterns and treatment rate. *Dis Aquat Organ, 60*(2), 123-131. doi:10.3354/dao060123

Gunnarsson, G. S., Blindheim, S., Karlsbakk, E., Plarre, H., Imsland, A. K., Handeland, S., . . . Nylund, A. (2017a). *Desmozoon lepeophtherii* (microsporidian) infections and pancreas disease (PD) outbreaks in farmed Atlantic salmon (*Salmo salar* L.). *Aquaculture, 468*, 141-148. doi:https://doi.org/10.1016/j.aquaculture.2016.09.035

Gunnarsson, G. S., Karlsbakk, E., Blindheim, S., Plarre, H., Imsland, A. K., Handeland, S., . . . Nylund, A. (2017b). Temporal changes in infections with some pathogens associated with gill disease in farmed Atlantic salmon (*Salmo salar* L). *Aquaculture, 468*, 126-134. doi:https://doi.org/10.1016/j.aquaculture.2016.10.011

Haugland, G. T., Olsen, A.-B., Rønneseth, A., & Andersen, L. (2017). Lumpfish (*Cyclopterus lumpus* L.) develop amoebic gill disease (AGD) after experimental challenge with *Paramoeba perurans* and can transfer amoebae to Atlantic salmon (*Salmo salar* L.). *Aquaculture, 478*, 48-55. doi:https://doi.org/10.1016/j.aquaculture.2016.04.001

Haugland, O., Mikalsen, A. B., Nilsen, P., Lindmo, K., Thu, B. J., Eliassen, T. M., . . . Evensen, O. (2011). Cardiomyopathy syndrome of atlantic salmon (*Salmo salar* L.) is caused by a double-stranded RNA virus of the Totiviridae family. *Journal of virology, 85*(11), 5275-5286. doi:10.1128/jvi.02154-10

Herrero, A., Thompson, K. D., Ashby, A., Rodger, H. D., & Dagleish, M. P. (2018). Complex Gill Disease: an Emerging Syndrome in Farmed Atlantic Salmon (*Salmo salar* L.). *Journal of Comparative Pathology, 163*, 23-28. doi:https://doi.org/10.1016/j.jcpa.2018.07.004

Hodneland, K., & Endresen, C. (2006). Sensitive and specific detection of Salmonid alphavirus using real-time PCR (TaqMan®). *Journal of Virological Methods, 131*(2), 184-192. doi:https://doi.org/10.1016/j.jviromet.2005.08.012

Hoffman, G. L., Dunbar, C. E., Wolf, K., & Zwillenberg, L. O. (1969). Epitheliocystis, a new infectious disease of the bluegill (*Lepomis macrochirus*). *Antonie Van Leeuwenhoek, 35*(2), 146-158. doi:10.1007/bf02219125

Hvas, M., Karlsbakk, E., Mæhle, S., Wright, D., & Oppedal, F. (2017). The gill parasite *Paramoeba perurans* compromises aerobic scope, swimming capacity and ion balance in Atlantic salmon. *Conservation Physiology, 5*. doi:10.1093/conphys/cox066

Hytterød, S., Andersen, L., Hansen, H., Blindheim, S., Poppe, T., Kristoffersen, A. B., & Mo, T. A. (2017a). AGD behandlingsstrategier - Dose-respons-studier med hydrogenperoksid og ferskvann. *Rapport 10*.

Hytterød, S., Andersen, L., Hansen, H., Blindheim, S. H., Poppe, T. T., Kristoffersen, A. B., & Mo, T. A. (2017b). AGD-behandlingsstrategier Dose-respons-studier med hydrogenperoksid og ferskvann.

Isaksen, T. (2013). *Ichthyobodo infections on farmed and wild fish - Methods for detection and identification of Ichthyobodo spp.*

Isaksen, T. E., Karlsbakk, E., Repstad, O., & Nylund, A. (2012). Molecular tools for the detection and identification of *Ichthyobodo* spp. (Kinetoplastida), important fish parasites. *Parasitology International, 61*(4), 675-683. doi:https://doi.org/10.1016/j.parint.2012.07.006

Isaksen, T. E., Karlsbakk, E., Watanabe, K., & Nylund, A. (2011). I*chthyobodo salmonis* sp. n. (Ichthyobodonidae, Kinetoplastida), an euryhaline ectoparasite infecting Atlantic salmon (*Salmo salar* L.). *Parasitology, 138*(9), 1164-1175. doi:10.1017/s0031182011000916

Jellett, J. F., & Scheibling, R. E. (1988). Virulence of *Paramoeba* invadens Jones (Amoebida, Paramoebidae) from Monoxenic and Polyxenic Culture1. *The Journal of Protozoology, 35*(3), 422-424. doi:https://doi.org/10.1111/j.1550-7408.1988.tb04121.x

Karlsbakk, E., Olsen, A. B., Einen, A.-C. B., Mo, T. A., Fiksdal, I. U., Aase, H., . . . Hansen, H. (2013). Amoebic gill disease due to *Paramoeba perurans* in ballan wrasse (*Labrus bergylta*). *Aquaculture, 412-413*, 41-44. doi:https://doi.org/10.1016/j.aquaculture.2013.07.007

Karlsen, M., Nylund, A., Watanabe, K., Helvik, J. V., Nylund, S., & Plarre, H. (2008). Characterization of '*Candidatus* Clavochlamydia salmonicola': an intracellular bacterium infecting salmonid fish. *Environ Microbiol, 10*(1), 208-218. doi:10.1111/j.1462-2920.2007.01445.x

Kent, M., Sawyer, T., & Hedrick, R. (1988). *Paramoeba pemaquidensis* (Sarcomastigophora: Paramoebidae) infestation of the gills of coho salmon *Oncorhynchus kisutch* reared in sea water. *Diseases of Aquatic Organisms, 5*, 163-169.

Kim, W. S., Kong, K. H., Kim, J. O., Jung, S. J., Kim, J. H., & Oh, M. J. (2017). Amoebic gill disease outbreak in marine fish cultured in Korea. *J Vet Diagn Invest, 29*(3), 357-361. doi:10.1177/1040638717690783

Kindt, M. M. (2017). *Eksperimentell smitte med Paramoeba perurans og utvikling av amøbisk gjellesykdom hos Atlantisk laks (Salmo salar, L). Påvirker vanntemperaturen virulensen hos klonale isolater av amøben?* Univeristy of Bergen

Kube, P. D., Taylor, R. S., & Elliott, N. G. (2012). Genetic variation in parasite resistance of Atlantic salmon to amoebic gill disease over multiple infections. *Aquaculture, 364-365*, 165-172. doi:https://doi.org/10.1016/j.aquaculture.2012.08.026

Kudryavtsev, A., Pawlowski, J., & Hausmann, K. (2011). Description of *Paramoeba atlantica* n. sp. (Amoebozoa, Dactylopodida) - a Marine Amoeba from the Eastern Atlantic, with Emendation of the Dactylopodid Families. *Acta Protozoologica, 50*, 239-253.

Kvellestad, A., Falk, K., Nygaard, S. M., Flesja, K., & Holm, J. A. (2005). Atlantic salmon paramyxovirus (ASPV) infection contributes to proliferative gill inflammation (PGI) in seawater-reared *Salmo salar*. *Dis Aquat Organ, 67*(1-2), 47-54. doi:10.3354/dao067047

Kvåle, B. L. (2020). *Effekt av termisk avlusing på gjellehelse hos laks: Hos patogenfri laks og i kommersiell produksjon på Vestlandet.* University of Bergen,

Leef, M. J., Harris, J. O., Hill, J., & Powell, M. D. (2005). Cardiovascular responses of three salmonid species affected with amoebic gill disease (AGD). *J Comp Physiol B, 175*(7), 523-532. doi:10.1007/s00360-005-0020-4

Leef, M. J., Harris, J. O., & Powell, M. D. (2005). Respiratory pathogenesis of amoebic gill disease (AGD) in experimentally infected Atlantic salmon *Salmo salar*. *Dis Aquat Organ, 66*(3), 205-213. doi:10.3354/dao066205

Leef, M. J., Hill, J. V., Harris, J. O., & Powell, M. D. (2007). Increased systemic vascular resistance in Atlantic salmon, *Salmo salar* L., affected with amoebic gill disease. *J Fish Dis, 30*(10), 601-613. doi:10.1111/j.1365-2761.2007.00840.x

Lima, P. C., Taylor, R. S., & Cook, M. (2017). Pseudocyst formation in the marine parasitic amoeba *Neoparamoeba perurans*: a short-term survival strategy to abrupt salinity variation. *J Fish Dis, 40*(8), 1109-1113. doi:10.1111/jfd.12588

Lovy, J., Becker, J. A., Speare, D. J., Wadowska, D. W., Wright, G. M., & Powell, M. D. (2007). Ultrastructural Examination of the Host Cellular Response in the Gills of Atlantic Salmon, *Salmo salar,* with Amoebic Gill Disease. *Veterinary Pathology, 44*(5), 663-671. doi:10.1354/vp.44-5-663

Marcos-López, M., & Rodger, H. D. (2020). Amoebic gill disease and host response in Atlantic salmon (*Salmo salar* L.): A review. *Parasite Immunology, 42*(8), e12766. doi:https://doi.org/10.1111/pim.12766

Martinsen, K. H., Thorisdottir, A., & Lillehammer, M. (2018). Effect of hydrogen peroxide as treatment for amoebic gill disease in Atlantic salmon (*Salmo salar* L.) in different temperatures. *Aquaculture Research, 49*(5), 1733-1739. doi:https://doi.org/10.1111/are.13627

Masouleh, A. S., Sharifpour, I., & Arani, A. S. (2006). The aerobic bacterial flora of hatchery reared Caspian Sea sturgeon fingerlings. *Journal of Applied Ichthyology, 22*, 261-264.

Merrifield, D. L., & Rodiles, A. (2015). 10 - The fish microbiome and its interactions with mucosal tissues. In B. H. Beck & E. Peatman (Eds.), *Mucosal Health in Aquaculture* (pp. 273-295). San Diego: Academic Press.

Mitchell, S. O., & Rodger, H. D. (2011). A review of infectious gill disease in marine salmonid fish. *Journal of Fish Diseases, 34*(6), 411-432. doi:https://doi.org/10.1111/j.1365-2761.2011.01251.x

Mitchell, S. O., Steinum, T. M., Toenshoff, E. R., Kvellestad, A., Falk, K., Horn, M., & Colquhoun, D. J. (2013). '*Candidatus* Branchiomonas cysticola' is a common agent of epitheliocysts in seawater-farmed Atlantic salmon *Salmo salar* in Norway and Ireland. *Dis Aquat Organ, 103*(1), 35-43. doi:10.3354/dao02563

Mo, T., Hytterød, S., Olsen, A. B., & Hansen, H. (2015). Utvikling av amøbegjellesykdom (AGD) hos laks i tre oppdrettsanlegg i 2013-2014. *Norsk Fiskeoppdrett, 2015*, 2015.

Morrison, R. N., Crosbie, P. B., & Nowak, B. F. (2004). The induction of laboratory-based amoebic gill disease revisited. *J Fish Dis, 27*(8), 445-449. doi:10.1111/j.1365-2761.2004.00561.x

Morrison, R. N., Young, N. D., & Nowak, B. F. (2012). Description of an Atlantic salmon (*Salmo salar* L.) type II interleukin-1 receptor cDNA and analysis of interleukin-1 receptor expression in amoebic gill disease-affected fish. *Fish Shellfish Immunol, 32*(6), 1185-1190. doi:10.1016/j.fsi.2012.03.005

Munday, B. L., Foster, C. K., Roubal, F. R., & Lester, R. J. G. (1990). *Paramoebic gill infection and associated pathology of Atlantic salmon, Salmo salar and rainbow trout, Salmo gairdneri in Tasmania*. San Diego, California. https://www.cabdirect.org/cabdirect/abstract/19910875795

Munday, B. L., Zilberg, D., & Findlay, V. (2001). Gill disease of marine fish caused by infection with *Neoparamoeba pemaquidensis*. *Journal of Fish Diseases, 24*(9), 497-507. doi:https://doi.org/10.1046/j.1365-2761.2001.00329.x

Norwegian Directorate of Fisheries (2020). Atlantic salmon, rainbow trout and trout - Grow out production. Retrieved from https://www.fiskeridir.no/Akvakultur/Tall-og-analyse/Akvakulturstatistikk-tidsserier/Laks-regnbueoerret-og-oerret/Matfiskproduksjon

Nowak, B., Valdenegro-Vega, V., Crosbie, P., & Bridle, A. (2014). Immunity to Amoeba. *Developmental & Comparative Immunology, 43*(2), 257-267. doi:https://doi.org/10.1016/j.dci.2013.07.021

Nowak, B. F. (2012). *Neoparamoeba perurans*. In P. T. K. Woo & K. Buchmann (Eds.), *Fish parasites: pathobiology and protection* (pp. 1-18). Wallingford: CABI.

Nowak, B. F., & Archibald, J. M. (2018). Opportunistic but Lethal: The Mystery of *Paramoebae*. *Trends Parasitol, 34*(5), 404-419. doi:10.1016/j.pt.2018.01.004

Nowak, B. F., Bryan, J., & Jones, S. R. (2010). Do salmon lice, *Lepeophtheirus salmonis*, have a role in the epidemiology of amoebic gill disease caused by *Neoparamoeba perurans*? *J Fish Dis, 33*(8), 683-687. doi:10.1111/j.1365-2761.2010.01158.x

Nylund, A., Hansen, H., Brevik, Ø. J., Hustoft, H., Markussen, T., Plarre, H., & Karlsbakk, E. (2018a). Infection dynamics and tissue tropism of *Parvicapsula pseudobranchicola* (Myxozoa: Myxosporea) in farmed Atlantic salmon (*Salmo salar*). *Parasites & Vectors, 11*(1), 17. doi:10.1186/s13071-017-2583-9

Nylund, A., Kvenseth, A. M., & Isdal, E. (1998). A Morphological Study of the Epitheliocystis Agent in Farmed Atlantic Salmon. *Journal of Aquatic Animal Health, 10*(1), 43-55. doi:https://doi.org/10.1577/1548-8667(1998)010<0043:AMSOTE>2.0.CO;2

Nylund, A., Pistone, D., Trösse, C., Blindheim, S., Andersen, L., & Plarre, H. (2018b). Genotyping of *Candidatus* Syngnamydia salmonis (chlamydiales; Simkaniaceae) co-cultured in *Paramoeba perurans* (amoebozoa; Paramoebidae). *Archives of Microbiology, 200*(6), 859-867. doi:10.1007/s00203-018-1488-0

Nylund, A., Røed, M., Blindheim, S., Trösse, C., & Andersen, L. (2021). Experimental challenge of Atlantic salmon *Salmo salar* using clones of *Paramoeba perurans, P. pemaquidensis* and *Tetramitus* sp. *Diseases of Aquatic Organisms, 145*, 1-13.

Nylund, A., Watanabe, K., Nylund, S., Karlsen, M., Sæther, P. A., Arnesen, C. E., & Karlsbakk, E. (2008). Morphogenesis of salmonid gill poxvirus associated with proliferative gill disease in farmed Atlantic salmon (*Salmo salar*) in Norway. *Archives of Virology, 153*(7), 1299-1309. doi:10.1007/s00705-008-0117-7

Nylund, S., Andersen, L., Sævareid, I., Plarre, H., Watanabe, K., Arnesen, C. E., . . . Nylund, A. (2011). Diseases of farmed Atlantic salmon *Salmo salar* associated with infections by the microsporidian *Paranucleospora theridion*. *Diseases of Aquatic Organisms, 94*(1), 41-57. Retrieved from https://www.int-res.com/abstracts/dao/v94/n1/p41-57/

Nylund, S., Nylund, A. R. E., Watanabe, K., Arnesen, C. E., & Karlsbakk, E. (2010). *Paranucleospora theridion* n. gen., n. sp. (Microsporidia, Enterocytozoonidae) with a Life Cycle in the Salmon Louse (*Lepeophtheirus salmonis*, Copepoda) and Atlantic Salmon (*Salmo salar*). *Journal of Eukaryotic Microbiology, 57*(2), 95-114. doi:https://doi.org/10.1111/j.1550-7408.2009.00451.x

Nylund, S., Steigen, A., Karlsbakk, E., Plarre, H., Andersen, L., Karlsen, M., . . . Nylund, A. (2015). Characterization of '*Candidatus* Syngnamydia salmonis' (Chlamydiales, Simkaniaceae), a bacterium associated with epitheliocystis in Atlantic salmon (*Salmo salar* L.). *Archives of Microbiology, 197*(1), 17-25. doi:10.1007/s00203-014-1038-3

Oldham, T., Rodger, H., & Nowak, B. F. (2016). Incidence and distribution of amoebic gill disease (AGD) — An epidemiological review. *Aquaculture, 457*, 35-42. doi:https://doi.org/10.1016/j.aquaculture.2016.02.013

Olsvik, P. A., Lie, K. K., Jordal, A.-E. O., Nilsen, T. O., & Hordvik, I. (2005). Evaluation of potential reference genes in real-time RT-PCR studies of Atlantic salmon. *BMC Molecular Biology, 6*(1), 21. doi:10.1186/1471-2199-6-21

Page, F. C. (1987). The Classification of 'Naked' Amoebae (Phylum Rhizopoda). *Archiv für Protistenkunde, 133*(3), 199-217. doi:https://doi.org/10.1016/S0003-9365(87)80053-2

Parsons, H., Nowak, B., Fisk, D., & Powell, M. (2001). Effectiveness of commercial freshwater bathing as a treatment against amoebic gill disease in Atlantic salmon. *Aquaculture, 195*(3), 205-210. doi:https://doi.org/10.1016/S0044-8486(00)00567-6

Pennacchi, Y., Leef, M. J., Crosbie, P. B. B., Nowak, B. F., & Bridle, A. R. (2014). Evidence of immune and inflammatory processes in the gills of AGD-affected Atlantic salmon, *Salmo salar* L. *Fish & Shellfish Immunology, 36*(2), 563-570. doi:https://doi.org/10.1016/j.fsi.2013.12.013

Pfaffl, M. W. (2004). Quantification strategies in real-time PCR. *AZ of quantitative PCR, 1*, 89-113.

Plarre, H., Devold, M., Snow, M., & Nylund, A. (2005). Prevalence of infectious salmon anaemia virus (ISAV) in wild salmonids in western Norway. *Diseases of Aquatic Organisms, 66*(1), 71-79. Retrieved from https://www.int-res.com/abstracts/dao/v66/n1/p71-79/

Powell, M. D., Fisk, D., & Nowak, B. F. (2000). Effects of graded hypoxia on Atlantic salmon infected with amoebic gill disease. *Journal of Fish Biology, 57*(4), 1047-1057. doi:https://doi.org/10.1111/j.1095-8649.2000.tb02210.x

Powell, M. D., Nowak, B. F., & Adams, M. B. (2002). Cardiac morphology in relation to amoebic gill disease history in Atlantic salmon, *Salmo salar* L. *Journal of Fish Diseases, 25*(4), 209-215. doi:https://doi.org/10.1046/j.1365-2761.2002.00356.x

Powell, M. D., Parsons, H. J., & Nowak, B. F. (2001). Physiological effects of freshwater bathing of Atlantic salmon (*Salmo salar*) as a treatment for amoebic gill disease. *Aquaculture, 199*(3), 259-266. doi:https://doi.org/10.1016/S0044-8486(01)00573-7

Powell, M. D., Reynolds, P., & Kristensen, T. (2015). Freshwater treatment of amoebic gill disease and sea-lice in seawater salmon production: Considerations of water chemistry and fish welfare in Norway. *Aquaculture, 448*, 18-28. doi:https://doi.org/10.1016/j.aquaculture.2015.05.027

Quaglio, F., Perolo, A., Bronzatti, P., Gustinelli, A., Menconi, V., Cavazza, G., . . . Fioravanti, M. L. (2016). Nodular gill disease in farmed rainbow trout (*Oncorhynchus mykiss*) in Italy. *J Fish Dis, 39*(9), 1139-1142. doi:10.1111/jfd.12446

Ram, S., Khurana, S., Singh, R. P., & Khurana, S. B. (1992). Loss of some virulence factors of enterotoxigenic *Escherichia coli* on repeated subcultures. *Indian J Med Res, 95*, 284-287.

Roberts, S. D., & Powell, M. D. (2008). Freshwater bathing alters the mucous layer of marine Atlantic salmon *Salmo salar* L. *Journal of Fish Biology, 72*(7), 1864-1870. doi:https://doi.org/10.1111/j.1095-8649.2008.01853.x

Rodger, H. (2007). Gill disorders: an emerging problem for farmed Atlantic salmon (*Salmo salar)* in the marine environment? *Fish Veterinary Journal, 9*, 38.

Rodger, H. (2014). Amoebic gill disease (AGD) in farmed salmon (*Salmo salar*) in Europe. *Fish Veterinary Journal, 14*.

Rodger, H., Murphy, K., Mitchell, S., & Henry, L. (2011). Gill disease in marine farmed Atlantic salmon at four farms in Ireland. *Veterinary Record, 168*(25), 668-668.

Rogerson, A., & Laybourn-Parry, J. (1992). The abundance of marine naked amoebae in the water column of the Clyde estuary. *Estuarine, Coastal and Shelf Science, 34*(2), 187-196. doi:https://doi.org/10.1016/S0272-7714(05)80104-0

Røed, M. (2016). *Eksperimentell smitte med Paramoeba perurans og AGD utvikling hos Atlantisk laks (Salmo salar, L). En komparativ studie av virulens hos klonede isolater av P. perurans.* University of Bergen,

Simon, P. (2003). Q-Gene: processing quantitative real-time RT–PCR data. *Bioinformatics, 19*(11), 1439-1440. doi:10.1093/bioinformatics/btg157

Smirnov, A. V., Chao, E., Nassonova, E. S., & Cavalier-Smith, T. (2011). A Revised Classification of Naked Lobose Amoebae (Amoebozoa: Lobosa). *Protist, 162*(4), 545-570. doi:https://doi.org/10.1016/j.protis.2011.04.004

Småge, S. B., Brevik, Ø. J., Frisch, K., Watanabe, K., Duesund, H., & Nylund, A. (2017). Concurrent jellyfish blooms and tenacibaculosis outbreaks in Northern Norwegian Atlantic salmon (*Salmo salar*) farms. *PLOS ONE, 12*(11), e0187476. doi:10.1371/journal.pone.0187476

Sommerset, I., Bang Jensen, B., Bornø, G., Haukaas, A., & Brun, E., (red.). (2021). *Fiskehelserapporten 2020*. Retrieved from www.vetinst.no:

Song, H., Santi, N., Evensen, O., & Vakharia, V. N. (2005). Molecular determinants of infectious pancreatic necrosis virus virulence and cell culture adaptation. *Journal of virology, 79*(16), 10289-10299. doi:10.1128/JVI.79.16.10289-10299.2005

Songe, M. M., Thoen, E., Evensen, Ø., & Skaar, I. (2014). In vitro passages impact on virulence of *Saprolegnia parasitica* to Atlantic salmon, *Salmo salar* L. parr. *Journal of Fish Diseases, 37*(9), 825-834. doi:https://doi.org/10.1111/jfd.12175

Steigen, A., Nylund, A., Plarre, H., Watanabe, K., Karlsbakk, E., & Brevik, Ø. (2018). Presence of selected pathogens on the gills of five wrasse species in western Norway. *Diseases of Aquatic Organisms, 128*(1), 21-35.

Steinum, T., Kvellestad, A., Colquhoun, D. J., Heum, M., Mohammad, S., Grøntvedt, R. N., & Falk, K. (2010). Microbial and pathological findings in farmed Atlantic salmon *Salmo salar* with proliferative gill inflammation. *Dis Aquat Organ, 91*(3), 201-211. doi:10.3354/dao02266

Steinum, T., Kvellestad, A., Rønneberg, L. B., Nilsen, H., Asheim, A., Fjell, K., . . . Dale, O. B. (2008). First cases of amoebic gill disease (AGD) in Norwegian seawater farmed Atlantic salmon, *Salmo salar* L., and phylogeny of the causative amoeba using 18S cDNA sequences. *J Fish Dis, 31*(3), 205-214. doi:10.1111/j.1365-2761.2007.00893.x

Sveen, S., Øverland, H., Karlsbakk, E., & Nylund, A. (2012). *Paranucleospora theridion* (Microsporidia) infection dynamics in farmed Atlantic salmon *Salmo salar* put to sea in spring and autumn. *Dis Aquat Organ, 101*(1), 43-49. doi:10.3354/dao02464

Tan, C. K., Nowak, B. F., & Hodson, S. L. (2002). Biofouling as a reservoir of *Neoparamoeba pemaquidensis* (Page, 1970), the causative agent of amoebic gill disease in Atlantic salmon. *Aquaculture, 210*(1-4), 49-58.

Taylor, R., Kube, P., Muller, W., & Elliott, N. (2009a). Genetic variation of gross gill pathology and survival of Atlantic salmon (*Salmo salar* L.) during natural amoebic gill disease challenge. *Aquaculture, 294*, 172-179. doi:10.1016/j.aquaculture.2009.06.007

Taylor, R. S., Muller, W. J., Cook, M. T., Kube, P. D., & Elliott, N. G. (2009b). Gill observations in Atlantic salmon (*Salmo salar*, L.) during repeated amoebic gill disease (AGD) field exposure and survival challenge. *Aquaculture, 290*(1), 1-8. doi:https://doi.org/10.1016/j.aquaculture.2009.01.030

Taylor, R. S., Wynne, J. W., Kube, P. D., & Elliott, N. G. (2007). Genetic variation of resistance to amoebic gill disease in Atlantic salmon (*Salmo salar*) assessed in a challenge system. *Aquaculture, 272*, S94-S99. doi:https://doi.org/10.1016/j.aquaculture.2007.08.007

Thoen, E., Tartor, H., Amundsen, M., Dale, O. B., Sveinsson, K., Rønning, H. P., . . . Gjessing, M. C. (2020). First record of experimentally induced salmon gill poxvirus disease (SGPVD) in Atlantic salmon (*Salmo salar* L.). *Veterinary Research, 51*(1), 63. doi:10.1186/s13567-020-00787-9

Timmerhaus, G., Krasnov, A., Nilsen, P., Alarcon, M., Afanasyev, S., Rode, M., . . . Jørgensen, S. M. (2011). Transcriptome profiling of immune responses to cardiomyopathy syndrome (CMS) in Atlantic salmon. *BMC Genomics, 12*, 459. doi:10.1186/1471-2164-12-459

Toenshoff, E. R., Kvellestad, A., Mitchell, S. O., Steinum, T., Falk, K., Colquhoun, D. J., & Horn, M. (2012). A Novel Betaproteobacterial Agent of Gill Epitheliocystis in Seawater Farmed Atlantic Salmon (*Salmo salar*). *PLOS ONE, 7*(3), e32696. doi:10.1371/journal.pone.0032696

Tolås, I. V. (2012). *Lukket merdsystem - AquaDomen: Effekt på smittedynamikk.* University of Bergen,

Veríssimo, C. D. M., Maschio, V. J., Correa, A. P. F., Brandelli, A., & Rott, M. B. (2013). Infection in a rat model reactivates attenuated virulence after long-term axenic culture of *Acanthamoeba* spp. *Memorias do Instituto Oswaldo Cruz, 108*(7), 832-835. doi:10.1590/0074-0276130099

Visvesvara, G. S., Moura, H., & Schuster, F. L. (2007). Pathogenic and opportunistic free-living amoebae: *Acanthamoeba* spp., *Balamuthia mandrillaris, Naegleria fowleri*, and *Sappinia diploidea*. *FEMS Immunology & Medical Microbiology, 50*(1), 1-26. doi:https://doi.org/10.1111/j.1574-695X.2007.00232.x

Watanabe, K., Karlsen, M., Devold, M., Isdal, E., LitlabÃ□Â¸, A., & Nylund, A. (2006). Virus-like particles associated with heart and skeletal muscle inflammation (HSMI). *Diseases of Aquatic Organisms, 70*(3), 183-192. Retrieved from https://www.int-res.com/abstracts/dao/v70/n3/p183-192/

Wessel, Ø., Braaen, S., Alarcon, M., Haatveit, H., Roos, N., Markussen, T., . . . Rimstad, E. (2017). Infection with purified *Piscine orthoreovirus* demonstrates a causal relationship with heart and skeletal muscle inflammation in Atlantic salmon. *PLOS ONE, 12*(8), e0183781. doi:10.1371/journal.pone.0183781

Wiik, R., Andersen, K., Uglenes, I., & Egidius, E. (1989). Cortisol-induced increase in susceptibility of Atlantic salmon, *Salmo salar*, to *Vibrio salmonicida*, together with effects on the blood cell pattern. *Aquaculture, 83*(3), 201-215. doi:https://doi.org/10.1016/0044-8486(89)90033-1

Wiik, S. (2020). *Effekt av ferskvassbehandling på gjeller hjå laks (Salmo salar L.) med amøbegjellesjukdom (AGD): gjellepatologi, prevalens og tettleik av Paramoeba perurans.* University of Bergen,

Wiik-Nielsen, J., Mo, T. A., Kolstad, H., Mohammad, S. N., Hytterød, S., & Powell, M. D. (2016). Morphological diversity of *Paramoeba perurans* trophozoites and their interaction with Atlantic salmon, *Salmo salar* L., gills. *Journal of Fish Diseases, 39*(9), 1113-1123. doi:https://doi.org/10.1111/jfd.12444

Wright, D. W., Nowak, B., Oppedal, F., Crosbie, P., Stien, L. H., & Dempster, T. (2018). Repeated sublethal freshwater exposures reduce the amoebic gill disease parasite,

Neoparamoeba perurans, on Atlantic salmon. *J Fish Dis, 41*(9), 1403-1410. doi:10.1111/jfd.12834

Young, N. D., Crosbie, P. B., Adams, M. B., Nowak, B. F., & Morrison, R. N. (2007). *Neoparamoeba perurans* n. sp., an agent of amoebic gill disease of Atlantic salmon (*Salmo salar*). *Int J Parasitol, 37*(13), 1469-1481. doi:10.1016/j.ijpara.2007.04.018

Young, N. D., Dyková, I., Crosbie, P. B. B., Wolf, M., Morrison, R. N., Bridle, A. R., & Nowak, B. F. (2014). Support for the coevolution of *Neoparamoeba* and their endosymbionts, *Perkinsela* amoebae-like organisms. *European Journal of Protistology, 50*(5), 509-523. doi:https://doi.org/10.1016/j.ejop.2014.07.004

Young, N. D., Dyková, I., Snekvik, K., Nowak, B. F., & Morrison, R. N. (2008). *Neoparamoeba perurans* is a cosmopolitan aetiological agent of amoebic gill disease. *Dis Aquat Organ, 78*(3), 217-223. doi:10.3354/dao01869

Zilberg, D., Gross, A., & Munday, B. L. (2001). Production of salmonid amoebic gill disease by exposure to *Paramoeba* sp. harvested from the gills of infected fish. *Journal of Fish Diseases, 24*(2), 79-82. doi:https://doi.org/10.1046/j.1365-2761.2001.00271.x

Zilberg, D., & Munday, B. L. (2000). Pathology of experimental amoebic gill disease in Atlantic salmon, *Salmo salar* L., and the effect of pre-maintenance of fish in sea water on the infection. *Journal of Fish Diseases, 23*(6), 401-407. doi:https://doi.org/10.1046/j.1365-2761.2000.00252.x

7 Appendix

Table 7.1 Mean ct-values and prevalence for the different assay analyzed for in the samples from field. G = gill. K = kidney.

Assay	**Before treatment**			**Dead during treatment**			**Treated**			**Untreated**		
	Ct-value		%	CT-value		%	Ct-value		%	Ct-value		%
	Average	Range	Prevalence	Average	Range	Prevalence	Average	Range	Prevalence	Average	Range	Prevalence
EF1A (**G**)	14.9	14.0 – 16.1	-	13.3	10.5 – 15.2	-	15.4	13.9 – 16.5		14.6	13.2 – 15.9	-
EF1A (**K**)	14.8	13.7 – 16.8	-	16.4	14.1 – 18.8	-	14.7	13.0- 16.1		14.2	12.4 – 15.8	-
Salmonid alphavirus (**G**)	Neg	Neg	0	Neg	Neg	0	Neg	Neg	0	Neg	Neg	0
Salmonid alphavirus (**K**)	Neg	Neg	0	Neg	Neg	0	Neg	Neg	0	Neg	Neg	0
Infectious salmon anemia virus (**G**)	Neg	Neg	0	Neg	Neg	0	Neg	Neg	0	Neg	Neg	0
Infectious salmon anemia virus (**K**)	Neg	Neg	0	Neg	Neg	0	Neg	Neg	0	36.0	36.0	
Piscine orthoreovirus 1 (**G**)	26.0	22.0 – 31.4	100.0	26.1	22.2 – 30.0	100.0	25.7	22.5 – 30.9	100.0	25.3	22.1 – 30.0	100.0
Piscine orthoreovirus 1 (**K**)	25.2	21.3 – 30.5	100.0	24.5	20.6 – 32.6	100.0	23.5	18.4 – 30.3	100.0	25.3	21.9 – 30.3	100.0
Piscine orthoreovirus 3 (**G**)	Neg	Neg	0	-	-	-	-	-	-	-	-	-
Piscine orthoreovirus 3 (**K**)	Neg	Neg	0	-	-	-	-	-	-	-	-	-
Piscine myocarditis virus (**G**)	28.6	20.8 – 36.9	100.0	22.0	17.1 – 33.2	100.0	26.5	19.6 – 34.1	100.0	26.9	21.4 – 31.7	100.0
Piscine myocarditis virus (**K**)	25.5	19.9 – 37.2	93.3	22.3	18.3 – 33.6	100.0	22.7	16.2 – 31.3	100.0	25.6	20.5 – 30.6	100.0
Salmon gill poxvirus (**G**)	27.1	19.3 – 36.2	100.0	23.8	18.5 – 33.5	100.0	25.6	18.2 – 33.7	93.3	26.4	18.9 – 34.4	100.0
Infectious pancreatic necrosis virus (**G**)	Neg	Neg	0	34.6	34.6	0	Neg	Neg	0	Neg	Neg	0
Infectious pancreatic necrosis virus (**K**)	36.6	34.7 – 37.8	10.0	35.5	25.3 – 35.7	16.7	35.8	35.7 – 36.0	10.0	Neg	Neg	0
Paranucleospora theridion (**G**)	19.5	11.9 – 23.4	100.0	13.5	10.0 – 17.5	100.0	21.3	15.1- 29.0	100.0	21.1	12.2 – 27.0	100.0
Paranucleospora theridion (**K**)	20.1	12.8 – 28.3	100.0	17.1	14.8 – 22.8	100.0	21.5	16.9 – 25.5	100.0	19.9	11.5 – 26.1	100.0
Paramoeba perurans (**G**)	16.8	12.7 – 21.1	100.0	29.2	22.8 – 32.2	75.0	30.6	23.9 – 33.7	33.3	17.1	11.3 – 22.7	100.0
Ichthyobodo spp. (**G**)	24.1	19.4 – 28.6	96.7	26.3	20.2 – 31.0	60.7	28.7	24.8 – 30.7	20.0	23.6	16.9 – 32.2	100.0
Perkinsea parasite (**G**)	Neg	Neg	0	-	-	-	-	-	-	-	-	-
Perkinsea parasite (**K**)	Neg	Neg	0	-	-	-	-	-	-	-	-	-
Tetracapsuloides bryosalmonae (**G**)	Neg	Neg	0	-	-	-	-	-	-	-	-	-
Tetracapsuloides bryosalmonae (**K**)	36.1	36.1	3.3	Neg	Neg	0	34.0	28.7 – 38.5	20.0	35.7	34.6 – 36.8	6.7
Parvicapsula pseudobranchicola (**G**)	Neg	Neg	0	31.5	29.1 – 33.3	17.9	33.2	32.5 – 33.9	6.7	32.5	31.0 – 34.0	6.7

Parvicapsula pseudobranchicola (**K**)	Neg	Neg	0	-	-	-	-	-	-	-	-	-
Candidatus Branchiomonas cysticola (G)	15.3	7.3 – 23.5	100.0	9.8	5.9 – 19.6	100.0	16.6	10.7 – 25.9	100.0	17.5	8.2 – 22.8	100.0
Candidatus Branchiomonas cysticola (K)	26.0	17.2 – 34.7	100.0	-	-	-	-	-	-	-	-	-
Candidatus Syngnamydia salmonis (G)	23.1	18.9 – 27.1	100.0	30.3	24.4 – 36.6	96.4	35.6	30.5 – 39.7	36.6	23.5	16.9 – 28.5	100.0
Candidatus Syngnamydia salmonis (K)	30.8	23.8 – 35.2	83.8	-	-	-	-	-	-	-	-	-
Tenacibaculum spp. (G)	29.2	26.9 – 31.5	13.3	31.0	25.0 – 34.5	25.0	32.5	30.1 – 36.6	20.0	34.6	27.3 – 37.6	46.7
Candidatus Piscichlamydia salmonis (G)	29.1	26.0 – 34.5	10.0	31.7	31.7 – 31.7	3.6	32.9	31.5 – 34.3	6.7	33.9	32.3 – 34.9	10.0
Tenacibaculum maritimum (K)	Neg	Neg	0	Neg	Neg	0	-	-	-	-	-	-

Table 7.3 Mean ct-values and prevalence for the assay Pperu analyzed for in the tissue samples from the challenge experiment.

	19 dpi			25 dpi			27 dpi			32 dpi		
	Ct-values		Prevalence	Ct-values		Prevalence	Ct-values		Prevalence	Ct-values		Prevalence
	Average	Range	%	Average	Range	%	Average	Range	%	Average	Range	%
Tank 1	35.9	35.9 – 35.9	10	Neg	Neg	0	Neg	Neg	0	Neg	Neg	0
Tank 2	Neg	Neg	0	Neg	Neg	0	Neg	Neg	0	Neg	neg	0
Tank 3	Neg	Neg	0	Neg	Neg	0	Neg	Neg	0	Neg	Neg	0
Tank 4	27.7	19.5 – 37.7.	100	30.4	30.4 – 30.4	50	31.5	28.4 – 34.6	70	Neg	Neg	0
Tank 5	Neg	Neg	0	Neg	Neg	0	Neg	Neg	0	Neg	Neg	0
Tank 6	34.9	33.6 – 36.2	40	Neg	Neg	0	Neg	Neg	0	Neg	Neg	0
Tank 7	Neg	Neg	0	Neg	Neg	0	Neg	Neg	0	Neg	Neg	0
Tank 8	Neg	Neg	0	Neg	Neg	0	Neg	Neg	0	Neg	Neg	0

Table 7.4 Mean ct-values and prevalence for the assay Perk analyzed for in the tissue samples from the challenge experiment.

	19 dpi			25 dpi			27 dpi			32 dpi		
	Ct-values		Prevalence	Ct-values		Prevalence	Ct-values		Prevalence	Ct-values		Prevalence
	Average	Range	%	Average	Range	%	Average	Range	%	Average	Range	%
Tank 1	38.6	38.6 – 38.6	20	37.8	37.8 – 37.8	50	37..6	35.0 – 38.7	40	-	-	-
Tank 2	37.1	36.0 – 38.1	40	37.3	37.3 – 37.3	50	37.4	37.2 – 27.5	20	-	-	-
Tank 3	38.8	38.6 – 39.0	40	33.7	33.7 – 33.7	50	38.4	38.4 – 38.4	10	-	-	-
Tank 4	29.7	26.0 – 33.5	40	37.2	37.2 – 37.2	50	36.3	36.0 – 37.2	40	-	-	-
Tank 5	Neg	Neg	0	Neg	Neg	0	38.8	38.8 – 38.8	10	-	-	-
Tank 6	37.8	37.0 – 38.6	40	37.6	37.3 – 38.0	100	36.9	36.6 – 37.3	30	-	-	-
Tank 7	37.4	36.8 – 38.0	40	37.4	37.4 – 37.4	50	37.0	37.0 – 37.0	10	-	-	-
Tank 8	37.3	37.2 – 37.3	40	Neg	Neg	0	Neg	Neg	0	-	-	-

Table 7.5 Mean ct-values and prevalence for the assay Sch analyzed for in the tissue samples from the challenge experiment.

	19 dpi			25 dpi			27 dpi			32 dpi		
	Ct-values		Prevalence	Ct-values		Prevalence	Ct-values		Prevalence	Ct-values		Prevalence
	Average	Range	%	Average	Range	%	Average	Range	%	Average	Range	%
Tank 1	Neg	Neg	0	35.1	33.8 – 36.5	100	36..9	36.9 – 36.9	10	34.5	32.8 – 36.1	9.1
Tank 2	36.9	36.7 – 37.1	40	Neg	Neg	0	36.5	36.5 – 36.5	10	35.1	33.2 – 36.1	15.8
Tank 3	Neg	Neg	0	Neg	Neg	0	Neg	Neg	0	34.6	33.0 – 36.1	10
Tank 4	32.3	25.8 – 36.8	60	Ne	Neg	0	Neg	Neg	0	33.7	31.1 – 36.3	10
Tank 5	Neg	Neg	0	Neg	Neg	0	35.5	34.0 – 37.6	30	35.8	35.8 – 35.8	4.5
Tank 6	33.2	32.5 – 33.7	80	36.6	36.6 – 36.6	50	37..3	37.3 – 37.3	10	33.6	31.1 – 36.9	19
Tank 7	37.1	36.9 – 37.4	40	28.8	28.8 – 28.8	50	35.3	35.3 – 35.3	10	Neg	Neg	0
Tank 8	Neg	Neg	0	Neg	Neg	0	Neg	Neg	0	34.0	30.9 – 36.8	14.3

Table 7.2 P-values for the positive assays from the field after the nonparametric Kruskal-Wallis test.

Pathogen	Before treatment vs. dead during treatment	Before treatment vs. treated	Before treatment vs. untreated	Dead during treatment vs. treated	Dead during treatment vs. untreated	Treated vs. untreated
P. perurans	<0.0001	<0.0001	>0.9999	>0.9999	<0.0001	<0.0001
Ichthyobodo spp.	0.0023	0.0763	>0.9999	>0.9999	0.0013	0.0579
Candidatus Syngnamydia salmonis	<0.0001	<0.0001	>0.9999	>0.9999	<0.0001	<0.0001
Candidatus Branchiomonas cysticola	0.0047	>0.9999	0.2296	0.0003	<0.0001	0.9756
Piscine myocarditis virus (gill)	0.0001	0.3284	>0.9999	0.1042	0.0025	>0.9999
Piscine myocarditis virus (kidney)	0.0001	0.3619	>0.9999	0.0927	<0.0001	0.0074
Salmon gill poxvirus	0.3527	0.2926	>0.9999	>0.9999	0.8537	0.7295
Paranucleospora theridion	<0.0001	>0.9999	0.4953	<0.0001	<0.0001	>0.9999
Piscine orthoreovirus 1	0.0568	>0.9999	>0.9999	0.0011	0.0078	>0.9999