#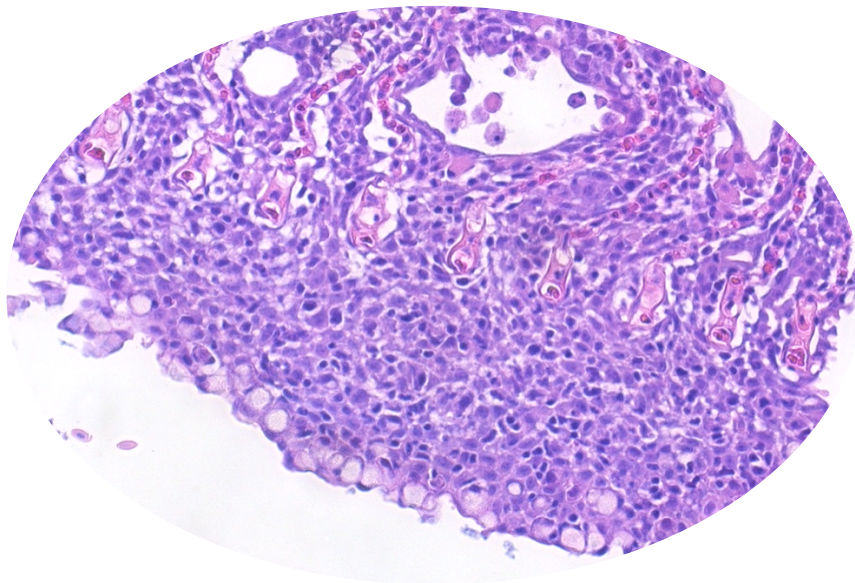 The effects of freshwater treatment and thermal delousing against Amoebic Gill Disease (AGD) and sea lice (*L. salmonis* and *C. elongatus*) on the gills of Atlantic salmon (*Salmo salar* L.), and experimental testing of the virulence of *Paramoeba perurans* with change in microbiota

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December 2022

2022

Paramoeba perurans (causative agent of AGD) and *Lepeophtheirus salmonis* in Norwegian aquaculture: Effects of freshwater treatment and thermal delousing against AGD and salmon louse on the gills of Atlantic salmon (*Salmo salar* L.) and experimental testing of *P. perurans* with different microbiota.

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Acknowledgements

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Abstract

The aim of this thesis was to study the effects of thermal and freshwater treatment against amoebic gill disease (AGD) and sea lice (*Lepeophtheirus salmonis,* Krøyer 1837 and *Caligus* spp.) on the gills of Atlantic salmon (*Salmo salar* L.). Gill diseases (GDs) and sea lice infestations are two of the main challenges in marine farmed salmon in Norwegian aquaculture. Treatments against these diseases represent a major cost to the industry, in addition to reducing the fish welfare due to stress and handling prior to treatment. The use of freshwater treatment has shown to reduce the levels of *Paramoeba perurans*, the causative agent of AGD, whereas thermal treatment is the main method to use against sea lice. GDs are often a complex problem caused by several pathogens, such as *Candidatus* Branchiomonas cysticola, *Ichthyobodo* spp., Salmon gill poxvirus, *Paranucleospora theridion, Paramoeba perurans,* etc., and are often referred to as complex gill disease (CGD) or proliferative gill inflammation (PGI). More knowledge about these gill pathogens and how mechanical treatments affect the gill health of farmed salmon is necessary in combating GDs and to better the fish health and welfare.

In this study, the gills were analysed for several pathogens (prevalence and density) before and after treatment and of the dead fish. At Location LA, typical AGD lesions were observed histologically, in addition to large amounts of fresh and bleedings aneurisms before and after treatment. The prevalence of PRV1, *P. theridion*, *Cand.* Branchiomonas cysticola, *Cand.* Piscichlamydia salmonis, *Cand.* Syngnamydia salmonis and *P. perurans* was 100 % at all sampling groups at this location. This shows that the freshwater treatment did not affect the prevalence of these pathogens. The mean number of lice was however significantly reduced from 0.44 louse before treatment to 0.05 louse after treatment, and the density of *P. perurans* was reduced. The average individual gill score was also significantly reduced using freshwater. The freshwater treatment resulted in a moderate increase in mortality during the treatment. At Location LB, salmon kept in cooled fresh water for 4 hours were subsequently put through a thermic treatment. The treatment did not result in a significant increase in mortality and the fish started eating shortly after treatment. The prevalence of *P. theridion*, PRV1 and *Cand.* Branchiomonas cysticola was 100 % before and after treatment, while the prevalence of *P. perurans* and *Cand.* Syngnamydia salmonis was significantly reduced after treatment. However, the average individual gill score at Location LB was not significantly reduced.

A challenge experiment was conducted to test the virulence of *P. perurans* cultured in different microbiota. Healthy Atlantic salmon smolts were challenged with a high virulent clone (H02/13Pp) or a low virulent clone (H20/16Pp) of *P. perurans* with high (HVBM) or low virulent (LVBM) bacteria medium. The groups challenged with H02/13Pp (HV-V.spl., HV-HVBM, HV-LVBM, HVBM-HV) had a significantly higher gill score than the groups challenged with H20/16Pp (LV-LVBM, LV-HVBM, HVBM-LV). The groups challenged with H20/16Pp (LV-LVBM, LV-HVBM, HVBM-LV) had higher

gill score than the two control groups (HVBM and MYA). By changing the bacterial composition in the culture media with *P. perurans*, reduced gill score was achieved. Loss of virulence in *P. perurans* is likely due to mutation in the amoeba or change in microbial community in the culture media.

Abbreviations

AGD	Amoebic gill disease
Cand. B. c.	*Candidatus* Branchiomonas cysticola
Cand. C. s.	*Candidatus* Clavichlamydia salmonicola
Cand. P. s.	*Candidatus* Piscichlamydia salmonis
Cand. S. s.	*Candidatus* Syngnamydia salmonis
CGD	Complex gill disease
CMS	Cardiomyopathy syndrome
PMCV	Piscine myocarditis virus
Ct-value	Cycle threshold value
Dpc	Days post challenge
E	Efficiency
EF1A	Assay for elongation factor from Atlantic salmon
Epit	Assay for *Candidatus* Branchiomonas cysticola
FDRG	Fish Disease Research Group
FHF	Fiskeri- og havbruksnæringens forskningsfinansiering (Norwegian Seafood Research Fund)
F-primer	Forward primer
R-primer	Reverse primer
GD	Gill disease
ILAB	The Industrial and Aquatic Laboratory, Thormøhlensgate 55, 5008 Bergen, Norway
ISAV	Infectious salmon anemia virus
IPNV	Infectious pancreatic necrosis virus
L	Liter
mL	Millilitre
µL	Microliter
MYA	Malt Yeast Agar
N	Number of individuals
NE	Normalized expression
NTC	Non template control
P. theridion	*Paranucleospora theridion*
O_2	Oxygen
P. perurans	*Paramoeba perurans*
PerL	*Perkinsela*-like symbiont
PGI	Proliferative gill inflammation
Epit	Epiteliocystis
Sch	*Candidatus* Syngnamydia salmonis
TB-Tuf	Assay for Tenacibaculum spp.
Pch	Assay for *Candidatus* Piscichlamydia salmonis
PKX	Assay for *Tetracapsuloides bryosalmonae*
Parvi	Assay for *Parvicapsula pseudobranchicola*
Costia	Assay for *Ichthyobodo* spp.
SGPV	Salmon gill poxvirus
PRV1	*Piscine orthoreovirus 1*
SAV	Salmonid Alphavirus

Real-time RT-PCR	Real time reverse transcriptase polymerase chain reaction
sp.	Species (unknown) within the genera
spp.	Species within the genera
UoB	University of Bergen
Hi-Tech Centre	The High Technology Center
L. salmonis	*Lepeophtheirus salmonis*
ASPV	Atlantic Salmon Paramyxovirus
FHF	The Norwegian Seafood Research Fund
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
K	Condition factor

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1 Introduction

1.1 Norwegian aquaculture

Since the 1970's, Norway has been among the world-leading producers of Atlantic salmon (*Salmo salar* L.), Rainbow trout (*Oncorhynchus mykiss*) and trout (*Salmo trutta*). The industry produces over 1 million tons of salmonids a year, and have the last few years focused on the expansion of the industry, making Norway the world's largest producer of Atlantic salmon (hereafter referred to as salmon, unless specified otherwise) (Dean et al. 2021; Gulla et al. 2020; Johansen et al. 2019; The Norwegian Directorate of Fisheries 2022). Due to the rapid industrial growth, many challenges regarding fish health, welfare and pathogens arise. According to The Health Situation in Norwegian Aquaculture 2021, gill diseases are one of the major health issues in on-growing salmon, in addition to mechanical injuries related to delousing and sea lice infestations (*Lepeophtheirus salmonis,* Krøyer 1837 and *Caligus* spp.) and other pathogens (bacteria and viruses). Sea lice and gill diseases can lead to osmoregulatory problems and increased mortality of the fish (Botwright et al. 2021; Fjelldal, Hansen, and Karlsen 2020; Hvas, Karlsbakk, Maehle, et al. 2017; Leef, Harris, and Powell 2005; Leef and Nowak 2013; Long, Garver, and Jones 2019). According to The Norwegian Directorate of Fisheries (2021), 64 million salmonids died during the marine production in 2021. Compared to 2018, the mortality of marine farmed salmonids has risen by 13.7 %, from 56.3 million individuals to 64 million individuals (The Norwegian Directorate of Fisheries, 2022). Mortality related to treatments and reduced growth are also a major cost to the industry (Nowak and Archibald 2018). For the Norwegian aquaculture industry to grow and ensure sustainable production, reducing fish mortality is crucial. Behavioural changes, morphological alterations, emaciation, and injuries are some of the important physical conditions that indicate poor welfare (Oliveira et al. 2021).

1.2 Gill diseases

Gill diseases (GDs) have influenced the Norwegian production of salmon since the mid 90's (Gunnarsson, Karlsbakk, et al. 2017; S. Nylund et al. 2011). The intimate contact between the gills and the aquatic environment exposes the gills to a range of pathogens and particulate matter, toxins, and biofouling organisms attached to the net of the sea cage (Bloecher et al. 2018; Herrero et al. 2018). The gills are responsible for critical physiological functions such as gas exchange, excretion of nitrogenous waste, osmoregulation, regulation of pH and hormone production (Evans, Piermarini, and Choe 2005; Herrero et al. 2018). Hence, disturbance of the gills is critical for the fish's physiological function. GDs are often referred to as complex gill diseases (CGDs) and have in many cases a multifactorial aetiology caused by multiple agents, causing a complex gill problem among farmed salmon. Both infectious and non-infectious agents can cause gill problems, including viruses, bacteria, parasites, phyto- and zooplankton species (Bloecher et al. 2018; Boerlage et al. 2020; Gjessing et al. 2019). However, GDs can also be caused by a single pathogen, such as the parasite *Paramoeba perurans (P. perurans)*, the

aetiological agent of amoebic gill disease (AGD) (Steinum et al. 2008; Neil D. Young et al. 2008). It can be challenging to identify the aetiological agents of GDs, and there are no efficient treatments available except against AGD (Gjessing et al. 2019; Herrero et al. 2018; Hvas, Karlsbakk, Maehle, et al. 2017). CGDs or GDs are often referred to as "proliferative gill inflammation" (PGI) or "proliferative gill disease" (PGD) in published articles (Boerlage et al. 2020; Herrero et al. 2018; Kvellestad et al. 2005; A. Nylund, A. K. Watanabe, et al. 2008; Rodger and Mitchell 2013). The pathological changes of the gills for all these terms are essentially the same, observed as inflammation, necrosis, hyperplasia of epithelial cells, and vascular changes in the secondary lamellae (Boerlage et al. 2020). GDs usually occur from mid-summer to the beginning of winter. Clinical signs of GDs in fish are non-specific, for example crowding against the net pen, swimming near the water surface, increased respiratory rate and/or reduced appetite. The gross pathology varies, but it often includes swollen gill filaments to some extent, accumulation of mucus on the gills, petechial haemorrhages, and pale gill filaments. Focal or diffuse gill lesions can be observed, often affecting several to all gill arches in affected individuals (Herrero et al. 2018).

1.2.1 Pathogens and GDs

Several pathogens are associated with GDs in farmed salmon. For instance, *Candidatus* Clavichlamydia salmonicola, *Saprolegnia* spp. and *Ichthyobodo necator* are some of the infectious agents that may cause gill problems during the freshwater phase (Ali 2005; Isaksen et al. 2010; Karlsen et al. 2008; Schmidt-Posthaus et al. 2012). After transfer to sea, the list of pathogens is even longer (Gjessing et al. 2017; S. Nylund et al. 2011); *Candidatus* Piscichlamydia salmonis (Draghi et al. 2004), *Candidatus* Branchiomonas cysticola (Mitchell et al. 2013), *Candidatus* Syngnamydia salmonis (Nylund et al. 2015), *Desmozoon lepeophtherii* (syn. *Paranucleospora theridion*) (S Nylund et al. 2011; Steinum et al. 2010)*, Paramoeba perurans, Ichthyobodo salmonis* (Isaksen et al. 2011) and Salmon Gill Poxvirus (SGPV) (S. Nylund, M. Karlsen, and Nylund 2008) are some of them. Atlantic Salmon Paramyxovirus (ASPV) (Kvellestad, Dannevig, and Falk 2003) has been associated with PGI, but more recent studies have shown that the virus is not consistently present with the disease (Fridell, Devold, and Nylund 2004; Gjessing et al. 2019; Herrero et al. 2018). Chlamydia-like organisms causing epitheliocystis are frequently observed as inclusions in the epithelial cells on the gills and are often associated with PGI. Epitheliocystis can be observed in fish as hypertrophic epithelial cells in areas with inflammatory cells and necrosis, resulting in circulatory disturbance. The cyst-like inclusions result in enlarged infected cells containing intravacuolar bacteria (Mitchell et al. 2010; Nylund et al. 2015). Although PGI and epitheliocystis often are associated, PGI has been used to describe outbreaks of GDs in farmed salmon during autumn, mostly affecting smolts during their first year at sea (Herrero et al. 2018). *P. theridion* is a microsporidian infecting both salmon and salmon louse. The parasite infects i.a. endothelium and epithelial cells of the skin and gills. This microsporidian appears at higher densities in salmon during autumn, compared to winter and spring time, and can also be associated with PGI (Gunnarsson,

Blindheim, et al. 2017; Weli et al. 2017). The poxvirus can occur in both freshwater and seawater and results in apoptosis of infected gill epithelial cells (Gjessing et al. 2017).

1.2.2 The gills

As mentioned above, the gills are a multifunctional organ and are covered and protected by the operculum (gill lid) (Evans et al. 2005; Haugarvoll et al. 2008). Salmon have four gill arches in addition to a semi degenerated gill, called pseudobranch. The gill arches are bearing the gills consisting of primary filaments with secondary lamellae on the upper- and bottom side of each filament. The secondary lamellae are the main site of gas exchange in fish gills due its large surface area and simple or bilayer squamous epithelium surface. The secondary lamellae consist of chloride cells, mucus cells, and pillar cells (specialised endothelium cells forming capillaries) (Figure 1.1) (Kryvi and Poppe, 2016). The blood stream in the lamellae and the surrounding water flows in the opposite directions. Due to diffusion of oxygen between the blood and water, the blood is oxygenated. This counter current system is extremely efficient (Koppang, Kvellestad, and Fischer 2015; Sandblom and Gräns 2017). The function of the pseudobranch is still somewhat unclear, but it is known that oxygenated blood from the organ is transferred to retina in the eye (Kryvi & Poppe, 2016).
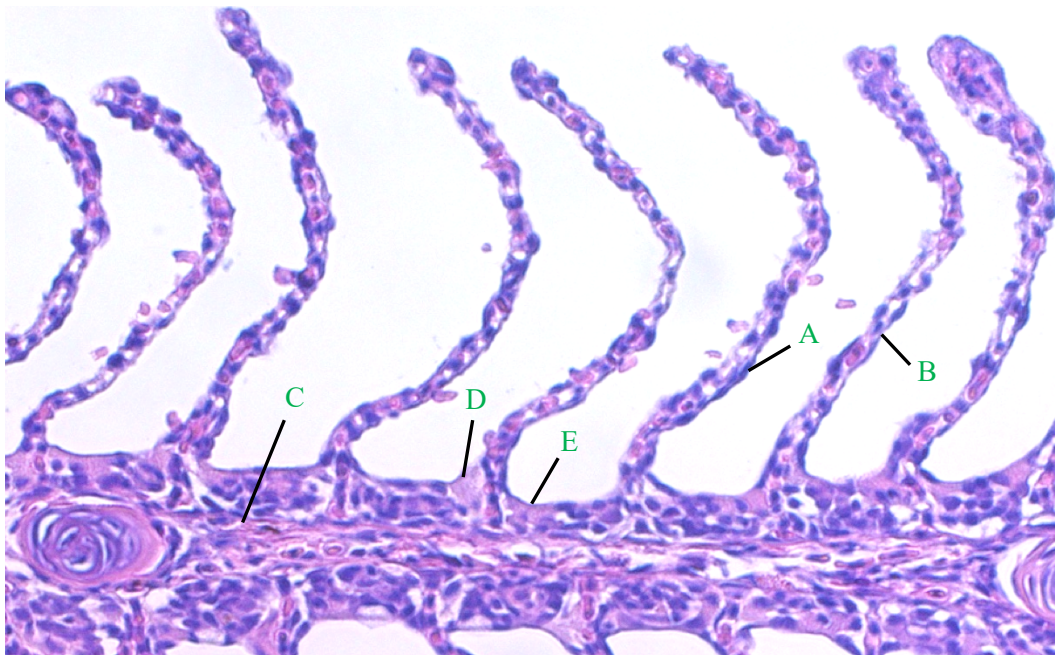
Figure 1.1: *Microscopic picture of secondary lamellae in Atlantic salmon. A: Epithelial cell. B: Pillar cell. C: Primary filament. D: Mucus cell. E: Chloride cell. The histological section is taken from fish after treatment at Location LB.*

1.3 Amoebic gill disease

Amoebic gill disease (AGD) in Atlantic salmon was originally discovered and described in Tasmania, Australia, in the mid-80s, causing severe economic losses to the Tasmanian production of salmon. Since

its discovery in the 1980s, AGD has spread to farmed fish species across the world (Chile, South-Africa, the US and Washington State, Spain, France, etc.) (Hjeltnes, Karlsbakk, To Atle Mo, et al. 2014; Wiik-Nielsen et al. 2016). The first case of AGD in farmed salmon in Norway was during autumn in 2006 at four sites, and was likely related to warmer temperatures, as the seawater was 3.5 °C higher than average temperatures before the disease outbreaks (Hvas, Karlsbakk, Maehle, et al. 2017; Steinum et al. 2008). After 2006, the disease did not occur until 2012, and has since then been a yearly challenge for gill health in Norwegian marine farmed salmon (Akhlaghi et al. 1996).

1.3.1 Causative agent of AGD: *Paramoeba perurans*

Paramoeba perurans (syn. *Neoparamoeba perurans*) is a free-living, amphizoid, opportunistic protist and ectoparasite infecting the gills of a range of fish species that is a global problem in salmonid aquaculture (Dykova and Novoa 2001; Michael L. Kent, Sawyer, and Hedrick 1988; Santos et al. 2010; Neil D. Young et al. 2008). *P. perurans* was originally described as *Neoparamoeba perurans* in the genus *Neoparamoeba* by Young et al. (2007). This was in 2013 suppressed by Feehan et al., and *Neoparamoeba* was converted to *Paramoeba.* In the literature, *Neoparamoeba perurans,* is used synonymous to *Paramoeba perurans* (Hjeltnes, Karlsbakk, To Atle Mo, et al. 2014). As of 2022, *Paramoeba perurans* is classified in Phylum Amoebozoa, Subphylum Lobosa, Class Discosea, Subclass Flabellinia, Order Dpctylopodida, Family Paramoebidae and Genus *Paramoeba* (Hjeltnes, Karlsbakk, To Atle Mo, et al. 2014; Kudryavtsev, Pawlowski, and Hausmann 2011; Smirnov et al. 2011).

The causative agent of AGD was initially believed to be *Paramoeba pemaquidensis* (Clark and Nowak 1999; Dykova, Figueras, and Novoa 1995; M. L. Kent, Sawyer, and Hedrick 1988; Nylund et al. 2021). However, *P. pemaquidensis* failed to cause AGD in salmon in experiments and *P. perurans* was later characterized from the gills of farmed salmon in Australia and Norway (Nylund et al. 2021; Steinum et al. 2008; Neil D. Young et al. 2008; Young et al. 2007). Hereafter, multiple challenge experiments were able to induce AGD by infecting salmon with *P. perurans* (Crosbie et al. 2012; O. M. V. Dahle et al. 2020; Nylund et al. 2021).

1.3.2 Pathology of AGD

After interaction between the gills and *P. perurans,* acute necrosis and hyperplasia of epithelia in the gill filaments can occur and cause compensatory plaque of tissue containing inflammatory immune cells (eosinophilic granulocytes) (Lovy et al. 2007). Hyperplasia reduces functional gill surface area when accumulating mucus by inhibiting the excretion of carbon dioxide across the gills, which leads to persistent respiratory acidosis. The respiratory disturbance is however just a small part of the AGD-pathology; when acute cardiovascular compromise occurs, systemic hypertension develops, causing circulatory collapse that may result in death. Clinical signs of AGD are increased ventilation rate,

lethargy, and anorexia (Hvas, Karlsbakk, Mæhle, et al. 2017; Mark D Powell, Reynolds, and Kristensen 2015).

After attaching to the fish gills, *P. perurans* proliferates by clonal division. The amoeba excretes hydrolytic enzymes, causing detrimental damage to the gill membrane (Bakketeig et al. 2015; Ní Dhufaigh et al. 2021). The pathology involves fusion of the secondary lamellae, mucoid lesions, necrosis, and hyperplasia of the epithelial cells (Hjeltnes, Karlsbakk, Tor Atle Mo, et al. 2014; Karlsbakk et al. 2013; Ruane and Jones 2013; Wiik-Nielsen et al. 2016). The excretion of mucus can be observed as pale patches macroscopically on the gill lamellae and can be used in gill scoring as a tool to assess the severity of the disease (Richard S. Taylor et al. 2009). Due to these pathological changes of the gills, the disease has the potential to interfere with the crucial physiological functions the gills have (Hvas, Karlsbakk, Maehle, et al. 2017; Munday, Zilberg, and Findlay 2001). Hvas et al. (2017) found that AGD caused by *P. perurans* reduced the capacity for aerobic activity in Atlantic salmon, and thus interfere with growth, appetite, and in general survival. Histologically, AGD lesions are often observed as cavitations between the lamellas, containing amoeba and inflammatory cells. These cavitations have earlier been referred to as "vesicles" or "cysts" (Adams and Nowak 2001; Munday et al. 2001).

In more advanced AGD lesions, decreased numbers of chloride cells and infiltration of immune cells (neutrophils and macrophages) have been reported (Botwright et al. 2021; Chang et al. 2019; Marcos-López and Rodger 2020). Chloride cells are specialized cells present at the afferent edge on the secondary lamellae of the gills (Chang et al. 2019; van der Heijden et al. 1999; Perry 1997; Wilson and Laurent 2002). While there is still limited knowledge about the Atlantic salmon immune response to *P. perurans*, it has not been shown that the salmon can develop innate or adaptive immunity against AGD (Bridle, Morrison, and Nowak 2006; Pennacchi et al. 2014; Vincent, Morrison, and Nowak 2006; N. D. Young et al. 2008). However, certain studies have identified an upregulation of important immune response genes, such as TNFα, CD8 and CD4, MHC I and MHC IIα within AGD-affected tissue, while others have shown a downregulation or no change in these immune related genes (Bridle, Morrison, Cupit Cunningham, et al. 2006; Bridle, Morrison, and Nowak 2006; Morrison et al. 2007; Pennacchi et al. 2014). More research about understanding host response to AGD is indeed needed, as well as understanding the behaviour of *P. perurans*.

1.4 Sea lice (*L. salmonis* and *C. elongatus*)

Lepeophtheirus salmonis, the salmon louse, is an ectoparasitic crustacean infecting salmonids in seawater (Costello 2006; Todd et al. 2006). The Scottish louse (*Caligus elongatus*) is not host specific but infects salmon among many other species (Agusti-Ridaura et al. 2019). Salmon louse is the largest threat to salmonid aquaculture; according to The Health Situation in Norwegian Aquaculture (2021),

the main reason for mortality and reduced fish welfare in ongrowing farmed salmon is mechanical injuries due to delousing treatments. The life cycle of *L. salmonis* consists of 8 stages: Two naupliar stages, one copepodite stage (infective), two chalimus stages (fixed), two preadult stages and one adult stage (Eichner, Hamre, and Nilsen 2015; Hamre et al. 2013). The louse is extremely dependent during the naupliar and copepodite stages to find a host before their nutrition sac is empty. When attached to a host, the louse feed of the salmon's skin, mucus and blood (Grimnes and Jakobsen 1996; Kragesteen et al. 2021; Stien et al. 2005). At moderate to high infestations, louse induced injuries can result in skin erosion and lead to secondary infections, osmoregulatory failure, physical damage, and immunosuppression and chronic stress (Bowers et al. 2000; Grave et al. 2004; Mackinnon 1998; Mordue and Birkett 2009; Overton et al. 2019). Due to the high fish density in the sea cages, finding a host is not a problem for the lice (Aaen et al. 2015; Bui et al. 2017; Cerbule and Godfroid 2020; Jevne and Reitan 2019). Louse numbers are required by the Norwegian authorities to register at Norwegian fish farms. Counting of fixed, mobile and mature stages of sea lice is registered. If the mean louse number exceeds 0.5 adult female louse (0.2 during migration period) each fish, treatment must be conducted to keep the louse number as low as possible while considering the fish health and welfare (Overton et al. 2019).

1.5 Risk factors

Stress caused by environmental factors (e.g., temperature, pH, salinity, nitrogen compounds, presence of infectious agents) can negatively impact the immune system of salmon, thereby increasing their susceptibility to infections. Temperature is one of the main risk factors for salmon, as they are poikilothermic animals (Ottavia Benedicenti et al. 2019; Bowden 2008). For example, *L. salmonis* develops faster at 21 °C compared to 6 °C (Hamre et al. 2019). The same goes for AGD that usually occurs at temperatures above 12 °C. The first AGD-outbreak in Norway occurred when the sea temperature was 3.5 °C warmer than average temperatures (O. Benedicenti, Secombes, and Collins 2019; Hjeltnes, Karlsbakk, Tor Atle Mo, et al. 2014; Hvas, Karlsbakk, Mæhle, et al. 2017; Steinum et al. 2008). However, AGD-infections can occur at minimum temperature and salinity at 10.6 °C and 7.2 ppt (part per thousands), respectively. The infection pressure of fish diseases is therefore thought to increase with an increasing temperature (Sandvik et al. 2021). As for salinity levels, the amoeba thrives best in salinities > 32 ‰, as it is a marine organism (Bois et al. 2019; Johnson-Mackinnon, Oldham, and Nowak 1986; Oldham, Rodger, and Nowak 2016). Other environmental factors like bacterial dense and turbidity of organic matter may also affect the establishment of AGD (O. Benedicenti et al. 2019; Clark and Nowak 1999; Douglas-Helder et al. 2001; Douglas-Helders et al. 2003a).

1.6 Treatment methods

After countless treatments against salmon louse, Norwegian salmon farmers are experienced in handling cage-based bath treatments (Mark D. Powell, Reynolds, and Kristensen 2015). To know if the fish need treatment, monitoring of AGD and salmon louse is done by doing gill score and regularly counting of lice, respectively (Jensen et al. 2020; Taylor et al. 2016). The treatment methods against salmon louse are divided in medical and non-medical treatments. Medical treatment using chemicals (e.g., hydrogen peroxide, azamethiphos, delta/cypermethrin, emamectin, etc.) was more common a few years back, but due to resistance in the louse, non-medical methods must be used (Gharbi et al. 2015; Hannisdal et al. 2020; Overton et al. 2019). Cleaner fish (lump fish (*Cyclopterus lumpus*), ballan wrasse (*Labrus bergylta*), goldsinny wrasse (*Ctenolabrus rupestris*), and corkwing wrasse (*Symphodus melops*)) is used as biological control or treatment against sea lice, as they prey on the crustacean (Brooker et al. 2018; Hannisdal et al. 2020; Oliveira et al. 2021; Powell et al. 2018). The mechanical treatment methods preferred in the Norwegian salmonid aquaculture are use of freshwater, warm water (thermal delousing), flushing and brushing (Bui et al. 2022; Oliveira et al. 2021; Østevik et al. 2022).

Salmon louse and *P. perurans* are marine parasites and are thereby vulnerable to low salinities (< 25 ppt). This makes freshwater treatment an effective method of removing them (Bricknell et al. 2006; Hudson and Nowak 2021). Treatment against AGD using freshwater for 2-4 hours has become a common method of removing *P. perurans.* Alternatively, hydrogen peroxide (H_2O_2) can be used instead, even though freshwater treatment is more gentle on the gills and for the fish (Hjeltnes, Karlsbakk, Tor Atle Mo, et al. 2014). The oxidative disinfectant, H_2O_2, has been used since the 1990s against salmon louse in farmed salmon (Johnson, Constible, and Richard 1993; Kiemer and Black 1997; Pedersen 2019; Taylor et al. 2021; Urbina et al. 2019). However, the use of H_2O_2 reduces fish welfare due to disruption of the mucus layer when damaging the cells, leading to development of open wounds and lesions in the skin and make the fish more susceptible to other infectious agents (Overton et al. 2018; Vera and Migaud 2016). Additionally, the toxicity of H_2O_2 increases with water temperature (> 13 °C) (Adams, Crosbie, and Nowak 2012; Wynne et al. 2020).

After treatment, some amoebae survive and are still attached to the gills. AGD may reappear and repeated treatments within the same production cycle are often necessary (Adams et al. 2012; Clark, Powell, and Nowak 2003; Martinsen, Thorisdottir, and Lillehammer 2018; Parsons et al. 2001; Powell, Parsons, and Nowak 2001; Mark D. Powell et al. 2015; Taylor et al. 2021). Thermal delousing using warm water at 28-34 °C for 20-30 s has become the most common delousing method in salmonid aquaculture. This method is however controversial, as the crowding, pumping and exposure to elevated water temperatures may stress and inflict pain on the fish. After the handling and treatment, gill and brain haemorrhages have been observed, as well as aneurisms and thrombi on the gill filaments and loss

of skin and scales (Bui et al. 2022; Gismervik et al. 2019; Nilsson et al. 2019; Nordgreen et al. 2009; Østevik et al. 2022).

In addition to the high costs of treatments in regards of maintenance of well boats and increased fish handling, *L. salmonis* has been observed to tolerate variation in salinity, possibly making freshwater treatment a less efficient method in removing sea lice from marine farmed salmon (Andrews and Horsberg 2020; Ljungfeldt et al. 2017).

1.7 Microbiota

The knowledge about gill microbiome of farmed salmon in seawater is limited (Birlanga, 2022). Previous studies have suggested that environmental factors e.g., biofouling in salmon cages (Tan, Nowak, and Hodson 2002), the presence of lumpfish in net-pens with farmed salmon (Haugland et al. 2017), or microbial dysbiosis (microbial imbalance of the gill microbiome) (Nowak and Archibald 2018) can influence AGD-outbreaks at fish farms. Bacterial composition and organic matter have been shown to affect the virulence and density of *P. perurans* on the gills of salmon (O. Benedicenti et al. 2019; Bowman and Nowak 2004; O. M. v. Dahle et al. 2020; Dhufaigh et al. 2021; Slinger et al. 2021). As the gills are in intimate contact with the fish' external environment, the gills are exposed to a range of particles and microorganisms (bacteria, fungi, virus, microparasites) in the surrounding water (Herrero et al. 2018; Mitchell and Rodger 2011; Nowak and Archibald 2018). Bowman and Nowak (2004) found significant differences in bacterial community on the gills of Atlantic salmon in AGD-affected salmon and AGD-negative salmon. Most marine amoeba feed on bacteria, algae, or organic detritus, and it is therefore useful to gain a better understanding of the role of the bacterial community on the gills of salmon for future risk assessment of AGD (O. Benedicenti et al. 2019; Bovee et al. 1979).

1.8 Aim of the study

The aim of this study is to investigate if production thermal delousing and freshwater treatment against AGD affect the gill health of Atlantic salmon. The virulence of *P. perurans* cultured with different microbiota was also tested.

Hypothesis 1 (H_0): Freshwater treatment of salmon infected with *Paramoeba perurans* will not have a negative effect on the gills of salmon.

Hypothesis 2 (H_0): Thermal delousing in combination with freshwater treatment will not affect the gills of salmon.

Hypothesis 3 (H_0): Change of microbiota in the clonal cultures of *P. perurans* will not affect the virulence of the amoeba.

2 Materials and Methods

The material (gill- and kidney tissue) in this study is a part of a larger project financed by FHF (project number 901514). To outline the effects of treatment against AGD and salmon louse, material from two fish farms on the west coast of Norway (Figure 2.2) was collected (hypothesis 1 and 2). To test hypothesis 3, a challenge experiment was conducted at The Industrial and Aquatic Laboratory (ILAB) in collaboration with The Fish Disease Research Group (FDRG) at the University of Bergen (UoB) (FHF-project 901053). By doing this challenge, the aim was to test how bacteria affects the virulence of *P. perurans* clones.

The material from the challenge experiment and the two fish farms were analysed at UoB. The tissue samples for histology and real-time RT-PCR (reverse transcriptase polymerase chain reaction) were processed at FDRG's facilities at The High Technology Centre (Hi-tech Centre) in Bergen. The aim of this project is to gain knowledge that may prevent mortality and welfare challenges in relation to decreased gill health and suffering of farmed salmonids. In this master thesis, results from real-time RT-PCR of gills- and kidney tissue and histopathological analysis of the gills are represented and discussed.

2.1 Challenge experiment: Study of different microbiota with clonal cultures of *P. perurans* in development of AGD

The challenge experiment was conducted at ILAB's facilities at the Hi-tech Centre between October 28th and November 29th, 2021 (Figure 2.2). Atlantic salmon smolts were acclimatized in 9 experimental tanks (150 L), with 40 fish in each tank. Smolts were sampled at 11 days (N = 10), 18 days (N = 15), and at the termination of the experimental period (N = 15) 25 days post challenge (dpc). To study the interaction between salmon smolts, *P. perurans* and the "natural" bacteria media, disease-free salmon smolts were used in the challenge experiment. The average weight of the fish used in this experiment was 207 ± 25.4 grams, and the average length was 26 ± 0.9 cm. The *P. perurans* isolates and the bacteria cultures were obtained from the gills of salmon suffering of AGD at two fish farms located in Vestland County. The aim was to obtain knowledge about the possible importance of microbiota associated with clones of *P. perurans* during exposure of the smolts.

The challenge experiment was approved by The Norwegian Food Safety Authority (application 28053: *Mapping the significance of bacteria for the development of AGD after infection with P. perurans*).

2.1.1 *Paramoeba perurans* isolates

The *P. perurans* isolates used in the challenge were obtained from salmon suffering from AGD in Vestland County in 2013 (Isolate H02/13Pp) and 2016 (Isolate H20/16Pp). The *P. perurans* clones were

maintained in their original bacterial media after isolation from the gills of salmon and after conducting the challenge experiment. The H20/16Pp-isolate was passaged 101 times prior to challenge (28.10.21), while H02/13Pp was passaged ~ 170 times (L. Andersen, pers. com.) before the challenge was conducted. H02/13Pp was a highly virulent (HV) amoeba clone and H20/16Pp was a low virulent (LV) clone. The HV and LV clonal isolates were kept in continuous cultures together with the bacteria that came with the fish during isolation.

2.1.2 Infection and sampling of smolts with *P. perurans*

The salmon smolts sampled in this experiment were produced at ILAB. Daily inspections monitored fish welfare, fish behaviour, and water quality. Fish with signs of illness (e.g., wounds) or abnormal behaviour (e.g., irregular swimming pattern, balancing problems, swimming near the wall or surface, abnormal reactions, hyperventilation, etc.) were removed from the tank and euthanized. The fish had continuous access to food throughout the challenge. The fish were acclimatized to the test conditions in the tanks one week prior to the experiment.

The fish were sedated with Aqui-S (4 ml/m^3) and transferred from aqua hall to experimental tanks at ILAB. After acclimatization of the fish, they were bath challenged by adding bacteria and amoebae in the tanks (Figure 2.1). Throughout the challenge, the water temperature in the tanks was maintained at 16 °C and 12L:12D lighting conditions, water flow was 300 L/hour, salinity 34 ‰, and > 82 % oxygen saturation. The fish were challenged for one hour. Compressed air and/or oxygen was added to the tanks (depending on biomass of the fish) to ensure good water quality. 5000 amoeba/L were added to each tank to infect the fish. During infection of the fish, the water volume in the tanks was 150 liters.
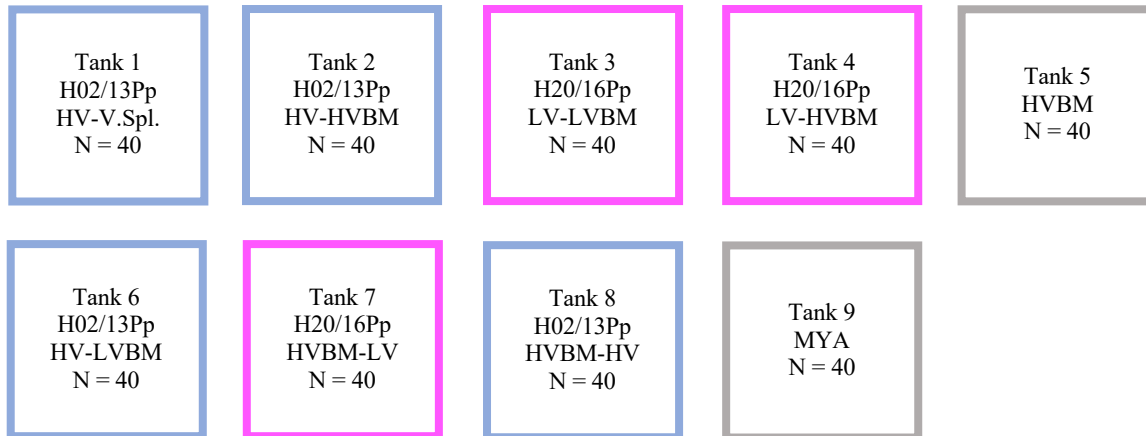
Tank 1 H02/13Pp HV-V.Spl. N = 40	Tank 2 H02/13Pp HV-HVBM N = 40	Tank 3 H20/16Pp LV-LVBM N = 40	Tank 4 H20/16Pp LV-HVBM N = 40	Tank 5 HVBM N = 40
Tank 6 H02/13Pp HV-LVBM N = 40	Tank 7 H20/16Pp HVBM-LV N = 40	Tank 8 H02/13Pp HVBM-HV N = 40	Tank 9 MYA N = 40	

Figure 2.1: *Schematic overview of the challenge set up (N = number of fish). Blue boxes represent the tanks containing the high virulent (HV) amoeba clones (H02/13Pp) and the pink boxes represent the low virulent (LV) amoeba clones (H20/16Pp). Grey boxes show the amoebae-free tanks. Date of challenge was 28.11.21. 5000 amoebae/liter in each tank, except from the two control groups (tank 5 and 9). HVBM = Normal media (obtained from the gills of farmed salmon during isolation of P. perurans in 2013). LVBM = Normal media (obtained from the gills of farmed salmon during isolation of P. perurans in 2016). MYA =Bacteria- and amoebae-free medium (Malt Yeast Agar).*

The fish in the challenge experiment were sampled at three dates after challenge: Ten fish were sampled from each tank at 11 dpc, whereas 15 fish were sampled from each tank at 18 and 25 dpc. The fish were euthanized prior to sampling by percussive stunning to the skull.

2.2 Treatment of AGD and delousing in the field

Freshwater treatment was used at site LA as treatment against AGD, while cooled freshwater followed by thermic treatment was used as treatment against AGD (*P. perurans*) and salmon louse at Location LB (Figure 2.2) in Vestland County in September 2021.

Location LA used freshwater treatment against AGD on 18.09.21 and 30 fish were sampled three days before treatment (15.09.21), 20 dead fish immediately following treatment, and 30 fish were sampled 11 days after treatment (29.09.21).

Location LB used cooled freshwater followed by thermic treatment against AGD and salmon lice on 08.09.21. 30 fish were sampled before treatment on 06.09.21, 30 dead fish immediately following treatment and 30 fish were sampled 17.09.21 (11 days after treatment).

The salmon were euthanized by an overdose of anaesthetics. The dead fish from both locations were frozen down and stored at -25 °C before sampling at the FDRG laboratory at the Hi-Tech Centre.

Sampling from the two fish farms follows the same principles: Samples were taken before and after treatment, in addition to samples of the dead fish. The dead fish were frozen down at -25 °C by the staff at the aquaculture site and were transported to the FDRG laboratories at UoB for later tissue sampling. The sampling included registration of mobile and fixed stages of salmon lice, gill scoring, tissue samples of the gills for histological examinations, and gill- and kidney tissue for real-time RT-PCR analysis. Samples the size of a matchhead of the apical part of the gills and kidney tissue were sampled for later RNA (Ribonucleic acid) extraction and real-time RT-PCR analysis. These samples were transferred to 2.0 mL Safe-Lock Tubes (Eppendorf) and stored at -25 °C until later analysis at UoB. The gill samples were taken from the second gill arch on the right side of the fish and the kidney samples from the head kidney.
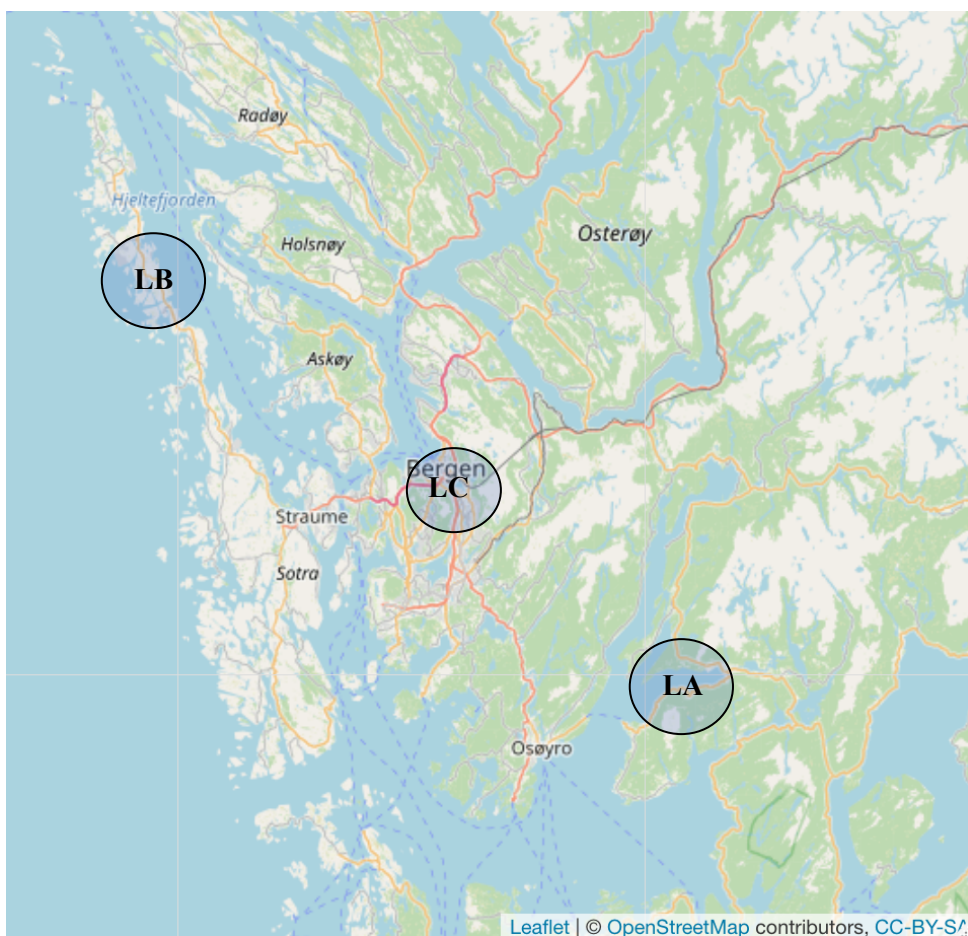
Figure 2.2: Approximate locations of the two fish farms (LA and LB) and ILAB (LC). The map was visualized in Rstudio using the "leaflet" function within the tidyverse package (Wickham et al. 2019).

2.2.1 Freshwater treatment: Location LA

There were 11 sea cages at Location LA. The location conducted freshwater treatment against AGD in all sea cages at the site. The fish farm was diagnosed with gill disease with detection of *Paramoeba perurans*. The farmed salmon were treated for four hours in freshwater at 14 °C on September 18th,

2021. The average length (L), weight (W), and condition factor (K) of the fish in this study is presented in Table 2.1. The sea temperature was 14.8 °C during treatment.

2.2.2 Mechanical delousing: Location LB

Location LB consists of six sea cages and carried out thermic treatment of salmon kept in cooled freshwater. The salmon were first treated with freshwater for four hours at 8 °C followed by thermal delousing (boat "Hordagut") at 30 °C for 30 s. The average length (L), weight (W), and condition factor (K) of the fish in this study is shown in Table 2.1. The average sea temperature the week of treatment (week 36) was 13.8 °C.

Table 2.1: *Average length (L), weight (W), and condition factor (K) of the fish before and after treatment and of the dead fish at Location LA and LB. cm = centimetres, g = grams. Location LA: N = 30 fish before treatment (15.09.21), N = 20 dead fish (18.09.21), N = 30 fish after treatment (29.09.21). Location LB: N = 30 fish before treatment (06.09.21), N = 30 dead fish (08.09.21), N = 30 fish before treatment (17.09.21).*

	Location LA			Location LB		
	Before treatment	**Dead fish**	**After treatment**	**Before treatment**	**Dead fish**	**After treatment**
L	53 ± 3.4 cm	47.7 ± 5.2 cm	54.1 ± 4.1 cm	44.3 ± 2.5 cm	39.5 ± 5.5 cm	44.7 ± 4.3 cm
W	1904.6 ± 5 g	1462.05 ± 585.2 g	2018.7 ± 435.6 g	1099.3 ± 184 g	770.4 ± 342.6 g	1141.4 ± 308.4 g
K	1.3 ± 0.06	1.3 ± 0.3	1.3 ± 0.09	1.3 ± 0.07	1.2 ± 0.37	1.2 ± 0.1

2.3 Gill scoring

The gill score system used in this study for grading the impact of *P. perurans* was developed by Taylor et al. (2009). The method is used to monitor the development of AGD by looking at gill lesions when sampling in the field and during challenge experiments. The gills of anaesthetised fish with white mucoid spots and hyperplastic areas (patches) on the gill surface are inspected (R. S. Taylor et al. 2009). Score "0" indicates healthy gills, whereas score "5" indicates heavily affected gills. During the challenge experiment, all 16 gill surfaces were scored (both front and back of the gill arches on the right and left side of the fish) according to Table 2.2. Only the gills on the left side of the fish were scored in the field (8 surfaces).

Table 2.2: *Gill score system to estimate the severity of AGD (R. S. Taylor et al. 2009).*

Infection level	Gill score	Gross description
Clear	0	No sign of infection and healthy red colour
Very light	1	One white spot, light scarring, or undefined neurotic 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 (>50 %)

2.3.1 Challenge experiment

The sampling from the challenge was done at the FDRG laboratories at UoB. The fish were kept in a tank containing Tricaine (Finquel Vet, > 135 mg/L) before sampling. The size of the gill tissues used for real-time RT-PCR was the size of a matchhead and was taken from the apical part of the second gill arch on the left side of the fish. Gill score was registered on both sides of the fish. The gill tissue was transferred to 2.0 mL Safe-Lock Tubes (Eppendorf) for real-time RT-PCR analysis. It was also taken backup samples using the rest of the second gill arch. These samples were transferred to 2.0 mL Nunc tubes (Thermo Scientific) and stored at -50 °C.

2.3.2 Field

Gill samples of the second gill arch on the left side of the fish were taken for histology at all samplings and were fixed in 10 % buffered formalin (Sigma-Aldrich) for later processing at UoB. Gill- and kidney tissue and registering of gill score were sampled of the dead fish at UoB. All backup samples of kidney and gills were stored in 2.0 mL Nunc tubes (Thermo Scientific) at -50 °C at the FDRG laboratories at UoB.

2.4 Histology

For histological analysis and assessment of the pathology associated with AGD, gill samples were fixed in 10 % buffered formalin and sent to Pharmaq Analytiq (Bergen) for preparation of histological sections. The tissue samples were prepared for microscopy by embedding them in paraffin and for histological analysis by using standard procedures (Bancroft and Gamble 2008). Histological sections of the gills were cut sagittally with a thickness of 2 μm and placed on coated slides of poly-L-lysine (SuperfrostPlus, Thermo Scientific, Germandy). After sectioning, the tissue slides were stained with hematoxylin and eosine (HE) using standard methods. The final sections were analysed at UoB.

Histological analysis of the gills before and after treatment included looking at possible changes of cell and tissue structures. A simplified version of the gill scoring system developed by Alf. S. Dalum (Pharmaq Analytiq) was used to identify the gill changes for this FHF-project. The histological analyses were based on five primary lamellae and each section was scored between 0 and 3 (Figure 2.3). Score 0 indicates healthy gills with no pathological changes, score 1 indicates mild changes (≤ 10 % pathological changes), score 2 moderate changes (10-50 % changes), and score 3 is extensive changes (> 50 % pathological changes of gill tissue). When scoring mucus cell hyperplasia, the average number of mucus cells on each secondary lamella was registered (calculated). ≤ 3 mucus cells indicate score 1, 5-8 mucus cells indicate score 2, and > 8 mucus cells indicate score 3. Thickened distal primary filament was given gill score based on affected filaments given in percentage. ≤ 10 % indicates score 1, 10-50 % indicates score 2, and > 50 % gills affected indicate score 3. Because only a small percentage of the total gill

tissue from each fish was studied, the average gill score for each pathological change was estimated. Visible pathogens on the gills were also registered.

The histological gill scoring was performed using Leica DM500 light microscope and Zeiss® Axio Scope A.1 with Axiocam 105 color camera. The images were processed in ZEN lite 2012 v.1.1.2.0.
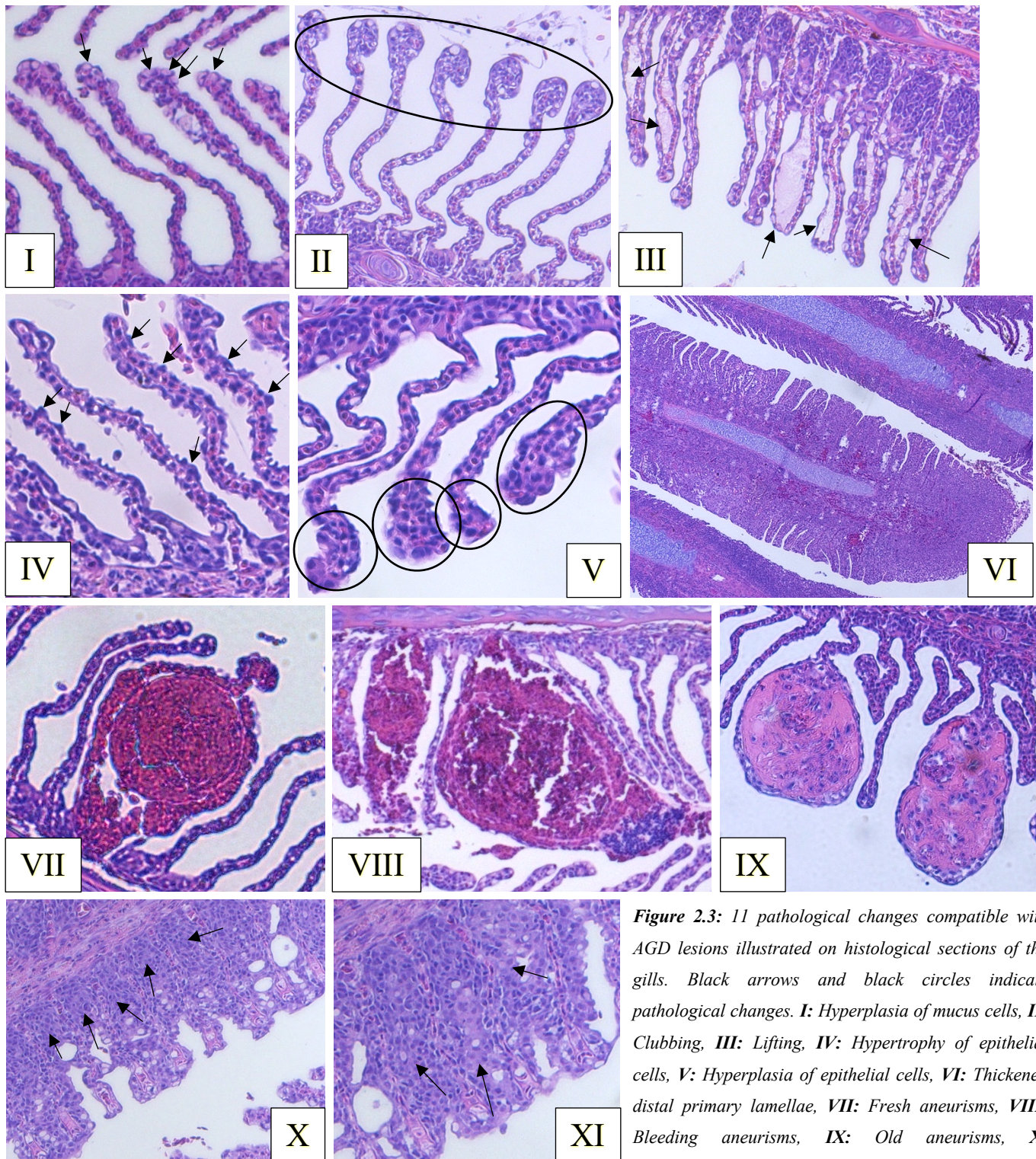
Figure 2.3: *11 pathological changes compatible with AGD lesions illustrated on histological sections of the gills. Black arrows and black circles indicate pathological changes. **I:** Hyperplasia of mucus cells, **II:** Clubbing, **III:** Lifting, **IV:** Hypertrophy of epithelial cells, **V:** Hyperplasia of epithelial cells, **VI:** Thickened distal primary lamellae, **VII:** Fresh aneurisms, **VIII:** Bleeding aneurisms, **IX:** Old aneurisms, **X:** Inflammation, **XI:** Necrosis.*

2.5 Detection of pathogens

2.5.1 RNA-extraction

RNA was isolated from gill- and kidney samples using TRIzol® Reagent (Life Technologies) for further real-time RT-PCR analysis, following the manufacturer's instructions (ThermoFisher Scientific 2020), with a few modifications.

Sterilized steel beads were first added to the samples containing gill or kidney tissue. In addition to the tissue samples, one negative extraction control (containing no tissue) was included after every ten tissue sample to detect any possible cross contamination between samples during the RNA-extraction. 1000 µL TRIzol was added to each sample tube (Eppendorf Safe Lock 2.0 mL). Tissue samples in these tubes were homogenized in a homogenizer (TissueLyser II Qiagen) for 3 minutes at 30 oscillations per second. After the homogenization, the samples were incubated in room temperature for 5-10 minutes and then spun down before 200 µL chloroform (Sigma-Aldrich) was added. After adding the chloroform, RNA was extracted by shaking the tubes heavily for 20 s and again incubated for 5 minutes in room temperature. Phase separation was achieved by centrifuging (Thermo Scientific™ Heraeus Fresco™) for 15 minutes at 12 000 x g at 4 °C. The mixture in the tubes was separated in a lower red phenol/chloroform layer, an interphase, and an upper aqueous phase. The upper aqueous phase (350 µL) was added to 500 µL isopropanol and mixed carefully. The RNA was left to precipitate for 10 minutes. After the incubation, the microtubes were centrifuged for 15 minutes at 12 000 x g and 4 °C, so that the RNA was precipitated as a pellet from the solution. The supernatant was decanted. The RNA-pellet was then washed with 1000 µL 75 % ethanol and a second time with 1000 µL 100 % ethanol by vortex (Vortex V-1 Plus, Biosan) for a few seconds. Between the washing of the RNA-pellets, the tubes were centrifuged for 5 minutes at 12 000 x g at 4 °C, so that the RNA pellet would stick to the wall of the tube when discarding the ethanol. After the 100 % ethanol was removed, the pellets were air dried for about 15 minutes (until the liquid had evaporated). RNAase free water (150 µL) (Sigma-Aldrich) that had been pre heated at 70 °C was added to dissolve the RNA. The purity and RNA-concentration from the RNA-isolation was controlled by using a spectrophotometer (Nanodrop™ 1000, Thermo Scientific). The Nanodrop detects absorbance at 260 nm (nucleic acid content), whereas absorbance at 280 nm indicates contamination with protein of the samples. A 260/280 ratio ~ 2 is considered pure. The samples were then stored at -25 °C until further real-time RT-PCR analysis.

2.5.2 Real-time RT-PCR

Real-time RT-PCR, also known as quantitative PCR (qPCR), is a sensitive molecular biological technique that is used to quantify specific RNA in samples with extracted RNA. The extracted RNA was analysed by using AgPath-ID™ One-Step RT-PCR Kit (ThermoFisher Scientific) and Applied Biosystems® QuantStudio™ Real-Time PCR System (ThermoFisher Scientific). Real-time RT-PCR

was used to detect RNA from specific pathogens such as *Paramoeba perurans,* Piscine myocarditis virus (PMCV), Salmonid Alphavirus (SAV), Infectious salmon anaemia virus (ISAV), etc. By using the one-step kit, it is possible to run both reverse transcription and the PCR-reaction in the same tube.

Primers (forward and reverse) and probes were made for eight specific assays as a 1:10 diluted solution. Primers, probes (TaqMan®), enzyme (25X RT-PCR, Ambion), buffer (2X RT-PCR, Applied Biosystems ®), and RNase free water (Sigma-Aldrich) was added to tubes, creating a mastermix for each assay (Table 2.3). After adding all the ingredients, the samples were vortexed and spun down. The mastermix was then added to a reaction plate consisting of 96 wells (Applied Biosystems® MicroAmp® Optical 96-Well Reaction Plate), adding 10.5 µL to each well. After doing so, 2 µL RNA-template was added to the wells. Negative isolation controls (RK) and one Non-Template Control (NTC) were also analysed for each assay. The RKs were used to detect possible contamination between samples during the RNA-extraction, and the NTCs were used to detect any contaminations in the real-time RT-PCR reagents. The wells were sealed with an adhesive film (MicroAmp™ Optical Adhesive Film, Applied Biosystem®). The plates were spun down for 15-20 s, and then analysed in QuantStudio™ 3 Real-Time PCR System.

Table 2.3: The mastermix components with associated volume (µL) for the assays.

Reagents	Each sample (µL)	Mastermix (µL)
2X Buffer	6.25	168.75
F primer	1.00	27.00
R primer	1.00	27.00
Probe	0.22	5.94
Enzyme	0.25	6.75
Water	1.78	48.06
Sum	10.50	283.50
RNA	2.00	
Total	12.50	

The real-time RT-PCR method shows how many amplification cycles it takes to reach the threshold (0.1 in this study). The amplification cycles are given as Ct (Cycle threshold)-values as a number of cycles until the fluorescence signal reaches the specific threshold value. Low Ct-values indicate high amount of target template in the sample, whereas high Ct-values indicate low amount of target template. Step one in the reaction is reverse transcription at 45 °C for 10 minutes. This transcribes the RNA to cDNA (complementary deoxyribonucleic acid). The temperature rises to 95 °C for 10 minutes to denaturate and inactivate the reverse transkriptase, and to activate the Taq DNA-polymerase. The two next steps are repeated 45 times for 15 s at 95 °C (DNA dissociation) and then annealing and elongation for 45 s. Fluorescence is quantitated at the end of each cycle. The ramp rates (heating and cooling) are set to 1.6 °C/s for the reactions, otherwise the amplification may fail.

Table 2.4 shows the pathogens tested for from the field experiment of the gill- and kidney samples. Elongation factor (EF1A) from Atlantic salmon was used as a reference gene (internal control). The gill samples from the challenge experiment were tested for EF1A, *P. perurans*, PerL and Sch.

Table 2.4: *Forward and reverse PCR primers and TaqMan® probes used in the Real-Time RT-PCR analysis to detect different pathogens. The efficiency of the assays is given in the references.*

Assay	Primer	Sequence	Reference
***Salmonid Alphavirus* (nsP1)**	Probe	CTG GCC ACC ACT TCG A	(Hodneland and Endresen 2005)
	Forward	CCG GCC CTG AAC CAG TT	
	Reverse	GTA GCC AAG TGG GAG AAA GCT	
***Infectious salmon anemia virus* (Segment 7)**	Probe	CAC ATG ACC CCT CGT C	(Platte et al. 2005)
	Forward	TGG GAT CAT GTG TTT CCT GCT A	
	Reverse	GAA AAT CCA TGT TCT CAG ATG CAA	
***Piscine orthoreovirus 1* (PRV1-M2)**	Probe	CTG GCT CAA CTC TC	(Nylund, Hansen, et al. 2018)
	Forward	CAA TCG CAA GGT CTG ATG CA	
	Reverse	GGG TTC TGT GCT GGA GAT GAG	
***Piscine myocarditis virus* (PMCV)**	Probe	TGG TGG AGC GTT CAA	(Nylund, Hansen, et al. 2018)
	Forward	AGG GAA CAG GAG GAA GCA GAA	
	Reverse	CGT AAT CCG ACA TCA TTT TGT GA	
***Infectious pancreatic necrosis virus* (IPNV)**	Probe	TCT TGG CCC CGT TCA TT	(Watanabe et al. 2006)
	Forward	ACC CCA GGG TCT CCA GTC	
	Reverse	GGA TGG GAG GTC GAT CTC GTA	
***Paranucleospora theridion* (*P. theridion*)**	Probe	TTG GCG AAG AAT GAA A	(Nylund et al. 2010)
	Forward	CGG ACA GGG AGC ATG GTA TAG	
	Reverse	GGT CCA GGT TGG GTC TTG AG	
***Paramoeba perurans* (*P. perurans*)**	Probe	CTG GTT CTT TCG RGA GC	(Nylund, Pistone, et al. 2018)
	Forward	GAT AAC CGT GGT AAA TCT AGA GCT AAT A	
	Reverse	TGG CAT TGG CTT TTG AAT CT	
***Tetracapsuloides bryosalmonae* (PKX)**	Probe	TGT TGT TAG GAT ATT TTC C	A. Nylund, pers. com.
	Forward	CAA GAT CGC GCC CTA TCA AT	
	Reverse	CGT CAC CCG TTA CAA CCT TGT	
Atlantic salmon elongation factor (EF1A)	Probe	ATC GGT GGT ATT GGA AC	(Olsvik et al. 2005)
	Forward	CCC CTC CAG GAC GTT TAC AAA	
	Reverse	CAC ACG GCC CAC AGG TAC A	
***Perkinsela*-like symbiont sp. (PerL)**	Probe	CGA AAG CTG AGG CTG T	Røed (2016)
	Forward	GGC ACT GCT CCC CTT CAA C	
	Reverse	CGA ACG TAC TTC CCC ATG A	
***Candidatus* Syngnamydia salmonis (Sch)**	Probe	TCC TTC GGG ACC TTA C	(Nylund et al. 2015)
	Forward	GGG TAG CCC GAT ATC TTC AAA GT	
	Reverse	CCC ATG AGC CGC TCT CTC T	
***Ichthyobodo* spp. (Costia)**	Probe	TCC ACG ACT GCA AAC GAT GAC G	(Isaksen et al. 2012)
	Forward	ACG AAC TTA TGC GAA GGC A	
	Reverse	TGA GTA TTC ACT TCC GAT CCA T	
***Tenacibaculum* spp.**	Probe	TTT CAA TAC ATA CAC CTC AGC	(Småge et al. 2018)
	Forward	AGT GTG ACG TCC ACC TT	
	Reverse	CTG TAA GCC AGG TTC TGT	
***Candidatus* Piscichlamydia salmonis (Pch)**	Probe	CAAAACTGCTAGACTAGAGT	(A. Nylund, K. Watanabe, et al. 2008)
	Forward	TCA CCC CCA GGC TGC TT	
	Reverse	GAATTCCATTTCCCCCTCTTG	
Salmon gill poxvirus (SGPV)	Probe	TTA TAC ACC ATC ACA TTT GTG	(Nylund et al. 2021)
	Forward	CAG AGG TTT TTC ATA CGC CAG AA	
	Reverse	GAG GTC ACG GTG ATG ACA GAA C	

2.6 Data analysis

2.6.1 Normalized expression values

The results from the real-time RT-PCR were normalized against ELA1A (reference gene for Atlantic salmon) to correct any differences in the amount of RNA in the analysed sample. E_{ref} is the effectivity of the elongation factor (ELA1A) and $Ct_{reference\ gene}$ is the Ct-value of the elongation factor. The effectivity of the relevant pathogen (E_{target}) was normalized with the corresponding Ct-value ($Ct_{target\ gene}$). Negative controls are not considered. The normalized expression value (NE) was calculated according to Eq. 1:

$$(1)\ NE_{Gill\ tissue} = \frac{(E_{ref})^{Ct_{reference\ gene}}}{(E_{target})^{Ct_{target\ gene}}}$$

To better illustrate the variation among the samples, the NE-values were converted to NE-fold. This was done by dividing the normalized expression value by the lowest expression value (NE_{min}) for gill- and kidney tissue at each location, using Eq. 2:

$$(2)\ NE_{fold} = \frac{NE}{NE_{min}}$$

NE-fold values give a large variation among the samples. Thus, the data was Log2 transformed to better illustrate the number of pathogens in each sample.

2.6.2 Density

Density indicates the number of pathogens each unit (area, volume or weight of infected tissue or organ) (Bush et al. 1997). The density can be used as a tool to measure the amount of a specific pathogen in one fish. In this study, the density was used to detect the amount of RNA from a specific pathogen in the analysed sample (volume). The density is expressed as normalized expression (NE) values and reversed Ct-values.

Reversed Ct-values were calculated according to Eq. 3 to better illustrate the relationship between the samples. This means that high values (low Ct-values) indicate high density, while low values (high Ct-values indicate) low density. This calculation was used when the Ct-value of the elongation factor was more or less stable. Undetermined samples (undetectable) were presented as 0 density.

$$(3)\ Density = 40 - Ct\ value$$

2.6.3 Prevalence

Prevalence is the quantity of infected individuals in a population, given in percent. The prevalence is calculated using Eq. 4 and gives an indication of the frequency of a pathogen in the studied population.

$$(4) \ Prevalence = \frac{Number \ of \ positive \ samples}{Total \ number \ of \ samples} \ x \ 100$$

2.6.4 Condition factor

The condition factor (K) of the fish was calculated by using the following formula:

$$(5) \ CF = \frac{W \ x \ 100}{L^3}$$

W = Weight of the fish (g), L = Length of the fish (cm).

2.6.5 Statistics

The NE-fold values of the positive individuals from each sampling were used for statistical analysis. The NE-fold values were used to study any changes in density of pathogens in the different groups and were not normal distributed. The Kruskal-Wallis H test, also called the "one-way ANOVA test", was therefore used to determine any statistically significant differences between the NE-fold values. This nonparametric test was followed by Dunn's multiple comparison test to detect which specific means are significant from other mean ranks from each group.

P-values are given in the appendix. Rstudio Desktop 2021.09.1+372 for macOS, version 11.6, was used to do the statistical analysis and to create the figures used in this thesis.

The figures shown in the results are visualized in Rstudio using the "ggplot2" function within the tidyverse package (Wickham et al. 2019).

3 Results

3.1 Challenge

3.1.1 Abiotic factors

The environmental factors were relatively constant throughout the challenge. The water temperature in the tanks was regulated to 16 °C (Figure 3.1) under 12L:12D lighting conditions, the salinity was set to 34 ‰ (Figure 3.2), and oxygen level was > 82 % (Figure 3.3).
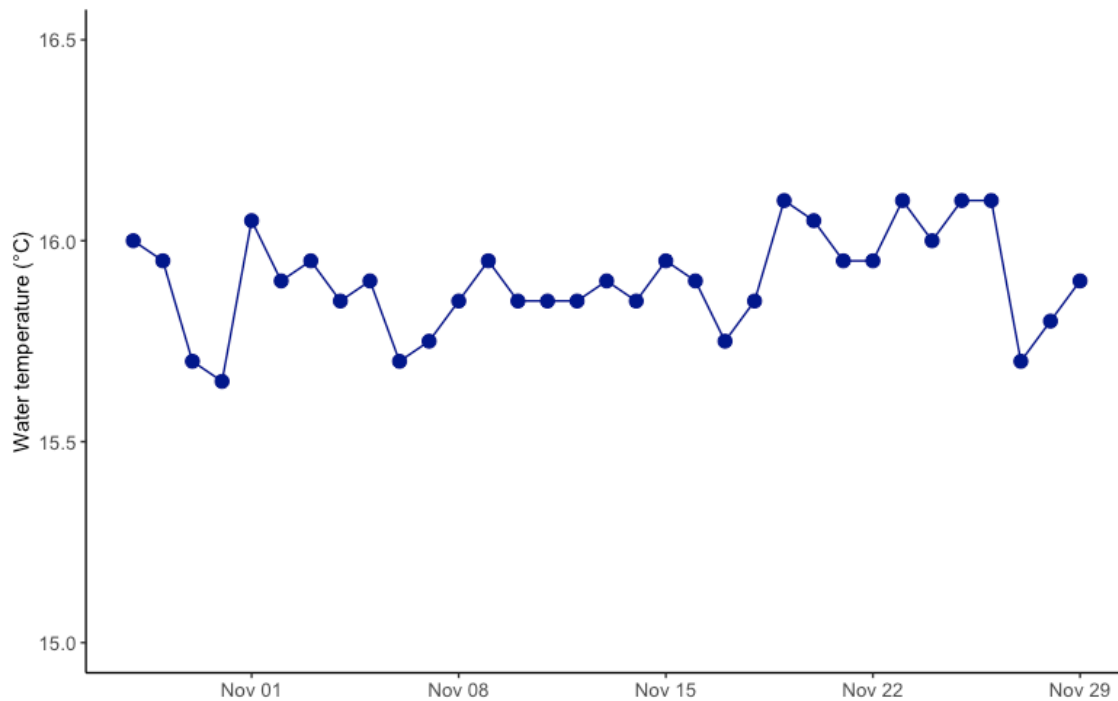

Figure 3.1: *Daily water temperature (°C) registered by the staff at ILAB during the challenge period (28.10.21-29.11.21). The average temperature throughout the challenge period was 15.9 ± 0.12 °C.*
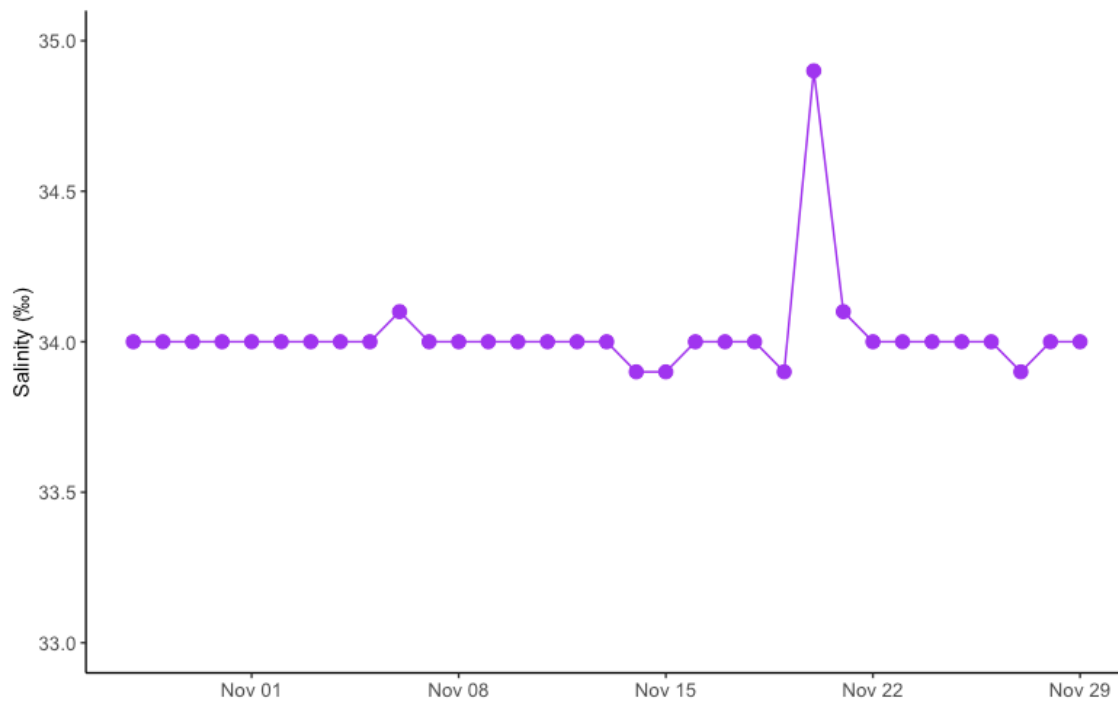
Figure 3.2: *Daily salinity (‰) registered by the staff at ILAB during the challenge period (28.10.21-29.11.21). The average salinity throughout the challenge period was 34 ± 0.2 %.*
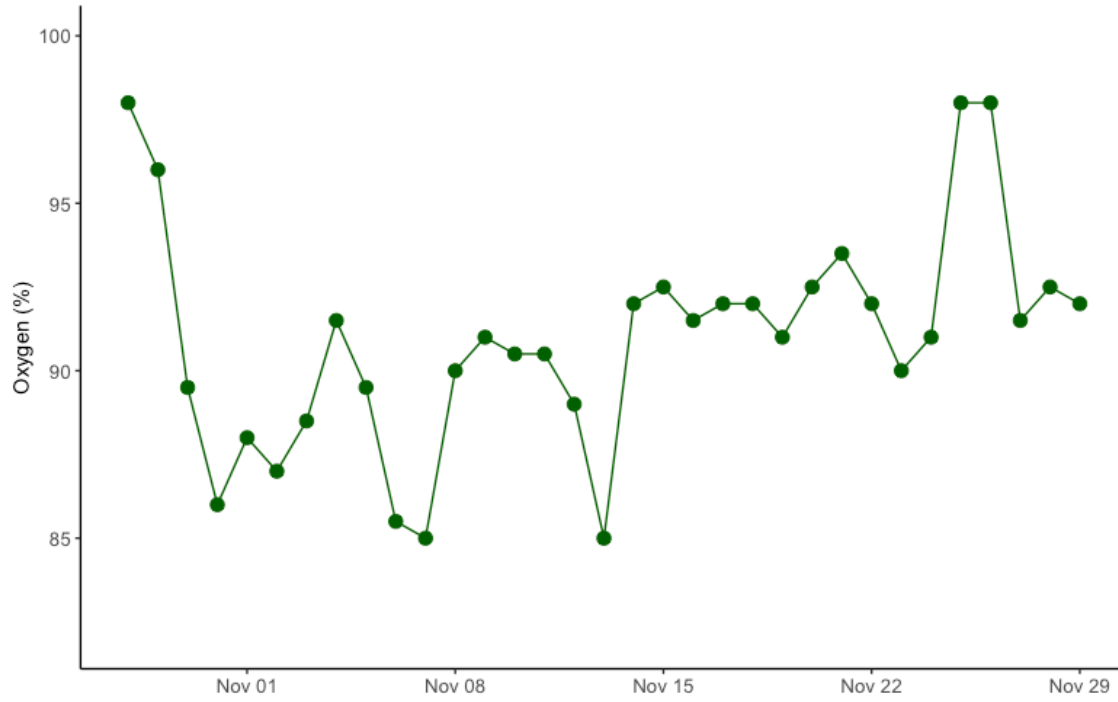

Figure 3.3: *Oxygen (%) registered daily by the staff at ILAB during the challenge period (28.10-2021-29.11.21). The average oxygen level throughout the challenge period was 91 ± 3.4 %.*

3.1.2 Biotic factors

One fish was registered dead (06.11.21) in tank 6 (HV-LVBM) during the challenge experiment. The average weight (g), length (cm) and condition factor (K) of the fish are presented in Table 3.1. The average weight and length show a slight increase throughout the challenge. The highest average K observed at 11 dpc was in tank 8 (HVBM-HV). The lowest average K observed at 11 dpc was 1.3 in tank 1 (HV-V.Spl.), tank 4 (LV-HVBM), tank 7 (HVBM-LV) and tank 9 (MYA). At 18 dpc, the average K was reduced in all tanks apart from in tank 7 (HVBM-LV). The K at 25 dpc increased from 18 dpc in tank 1 (HV-V.Spl.), tank 2 (HV-HVBM), tank 4 (LV-HVBM), tank 6 (HV-LVBM) and tank 9 (MYA), but was constant in tank 3 (LV-LVBM), tank 5 (HVBM), tank 7 (HVBM-LV) and tank 8 (HVBM-HV).

Table 3.1: Average weight (g), length (cm) and condition factor (K) of the fish in each tank at 11, 18 and 25 dpc (days post challenge).

	11 dpc			18 dpc			25 dpc		
	Weight (g)	Length (cm)	K	Weight (g)	Length (cm)	K	Weight (g)	Length (cm)	K
HV-V.Spl.	193.0 ± 27	25.3 ± 1.3	1.3 ± 0.1	216.3 ± 26	25.9 ± 1	1.2 ± 0.1	205.2 ± 19.6	26.6 ± 2.0	1.3 ± 0.2
HV-HVBM	184.4 ± 30.6	25.1 ± 1.4	1.4 ± 0.07	216.2 ± 24.3	26.2 ± 1.3	1.2 ± 0.1	193.4 ± 16.3	26.5 ± 1.4	1.4 ± 0.1
LV-LVBM	172.9 ± 32.2	24.3 ± 1.6	1.4 ± 0.09	210.2 ± 27.1	26.0 ± 1.2	1.3 ± 0.1	198.3 ± 10.1	26.6 ± 1.1	1.3 ± 0.1
LV-HVBM	190.8 ± 15.5	25.3 ± 0.7	1.3 ± 0.1	225.7 ± 38.4	26.4 ± 1.4	1.2 ± 0.1	202.0 ± 27.7	26.5 ± 1.6	1.3 ± 0.1
HVBM	184.8 ± 27.9	24.7 ± 1.4	1.4 ± 0.0	207.1 ± 20.2	25.6 ± 1.1	1.2 ± 0.1	227.2 ± 20.9	26.2 ± 0.9	1.2 ± 0.1
HV-LVBM	175.3 ± 29.2	24.4 ± 1.2	1.4 ± 0.07	217.8 ± 31.6	26.2 ± 1.3	1.2 ± 0.1	200.4 ± 13.2	26.6 ± 1.1	1.3 ± 0.1
HVBM-LV	203.5 ± 26.7	25.5 ± 1	1.3 ± 0.07	206.3 ± 31.5	25.9 ± 1.7	1.3 ± 0.01	214.1 ± 17.1	26.9 ± 1.2	1.3 ± 0.1
HVBM-HV	165.2 ± 23.8	24.3 ± 0.9	1.5 ± 0.1	211.2 ± 22.8	26.1 ± 1.3	1.2 ± 0.1	217.0 ± 24.1	26.9 ± 1.7	1.2 ± 0.1
MYA	192.4 ± 34.3	25.3 ± 1.4	1.3 ± 0.05	241.1 ± 28.3	26.9 ± 1.1	1.1 ± 0.1	202.0 ± 22.9	28.1 ± 1.4	1.3 ± 0.1

3.1.3 Gill score

Gill score (GS) was observed in all groups of salmon, except in the group exposed to HVBM. The group exposed to Malt Yeast Agar Medium (MYA) had a mean GS compatible with gills of disease-free salmon. The groups (HV-Vspl, HV-HVBM, HV-LVBM, HVBM-HV) challenged with the high virulent clone (H02/13Pp) of *P. perurans* had the highest gill scores (Figure 3.4). The fish group challenged with H02/13Pp cultured in the original bacteria medium (HV-HVBM) had a significant higher GS than the other groups (p = 0.017). GS in the other groups (LV-LVBM, LV-HVBM, HVBM-LV) challenged with the low virulent clone (H20/16Pp) had a higher GS than the two control groups but was significantly lower than the groups (HV-V.Spl., HV-HVBM, HV-LVBM, HVBM-LV) challenged with H02/13Pp (HV) (p = 0.012). It was not a significant difference in mean GS between the groups (LV-LVBM, LV-HVBM, HVBM-LV) challenged with H20/16Pp.
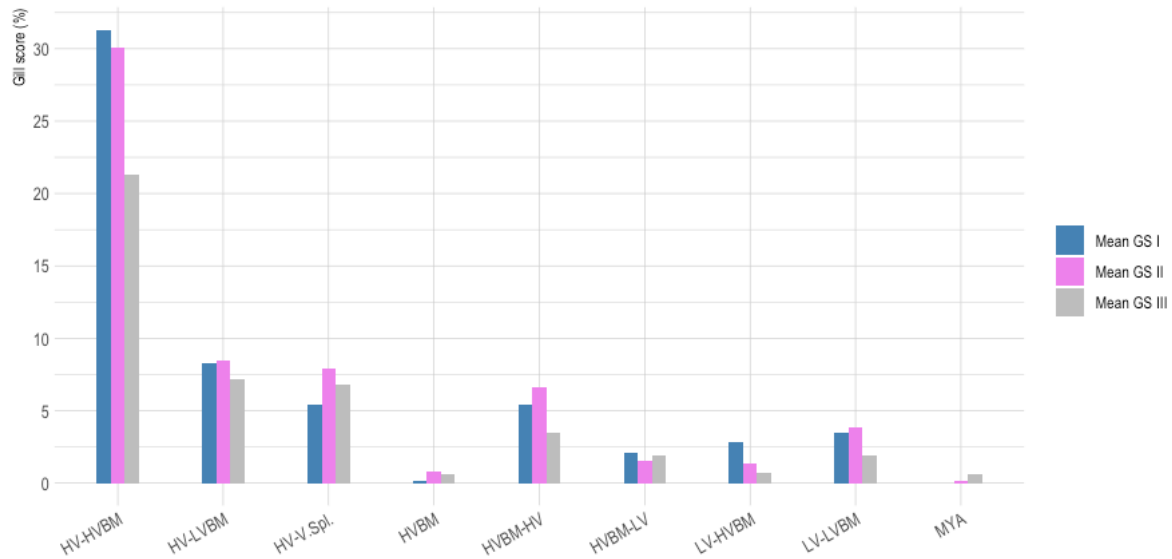
Figure 3.4: *Gill score (GS) of the fish in each tank from the challenge experiment. GS I = 11 dpc of P. perurans, GS II = 18 dpc of P. perurans, GS III = 25 dpc of P. perurans. GS is given in percent of maximum GS.*

3.1.4 Real-time RT-PCR

340 samples of gill tissue were analysed for *Paramoeba perurans, Perkinsela*-like symbiont and *Candidatus* Syngnamydia salmonis in the challenge experiment. The average and range of Ct-values and prevalence of *P. perurans* are presented in Table 3.2. The prevalence of *P. perurans* was 100 % throughout the challenge in the tanks challenged with H02/13Pp (HV-V.Spl.,HV-HVBM, (HV-LVBM, HVBM-HV). The prevalence of *P. perurans* in tank 4 (LV-HVBM) at 11 dpc was 70 % and was reduced to 0 % at 18 and 25 dpc. The prevalence in tank 5 (HVBM) increased from 0 to 46.7 % between 11 and 18 dpc. At 11 dpc, the prevalence in tank 9 (MYA) was 50 % but was reduced to 0 % at 18 and 25 dpc. Ct-values and prevalence for the *Perkinsela*-like symbiont and *Candidatus* Syngnamydia salmonis assays are given in the appendix.

Table 3.2: *Average and range of Ct-values and prevalence (%) of Paramoeba perurans at 11, 18 and 25 dpc (days post challenge).*

	11 dpc			18 dpc			25 dpc		
	Ct-value		Prevalence	Ct-value		Prevalence	Ct-value		Prevalence
	Average	Range	%	Average	Range	%	Average	Range	%
HV-V.Spl.	22.6	16.1 - 29.9	100	23.2	19.6 - 26.3	100	22.9	12.6 - 27.4	100
HV-HVBM	17.2	14.4 - 20.8	100	20.8	16.7 - 25.1	100	23.1	19.7 - 26.3	100
LV-LVBM	35.7	35.7 -	10	Neg	Neg	0	35.1	33.9 – 35.7	18.8
LV-HVBM	32.9	27.6 - 36.5	70	Neg	Neg	0	Neg	Neg	0
HVBM	Neg	Neg	0	35.8	33.6 - 37.5	46.7	Neg	Neg	0
HV-LVBM	21.8	16.8 - 25.8	100	22.7	18.4 – 27.5	100	24.6	19.4 – 27.9	100
HVBM-LV	35.0	33.5 – 36.5	20	35.6	33.7 - 38.6	86.7	Neg	Neg	0
HVBM-HV	23.6	15.9 - 28.7	100	26.5	20.5 - 34.7	100	24.8	19.8 - 27.9	100
MYA	35.0	33.3 - 36.4	50	Neg	Neg	0	Neg	Neg	0

The density (40 – Ct-value) of *P. perurans* is illustrated in Figure 3.5. Straight lines show the average density at 11, 18 and 25 dpc. The groups challenged with H02/13Pp (HV-V.Spl., HV-HVBM, HV-LVBM, HVBM-HV) had highest density at all sampling dates. The groups challenged with H20/16Pp (LV-LVBM, LV-HVBM, HVBM-LV) showed no or low density of *P. perurans* at 11 dpc. The fish challenged with HVBM were negative for *P. perurans* at 11 and 25 dpc. At 18 dpc, the fish were negative for *P. perurans* in tank 3 (LV-LVBM), tank 4 (LV-HVBM) and tank 9 (MYA). The fish in tank 4 (LV-HVBM), tank 5 (HVBM), tank 7 (HVBM-LV) and tank 9 (MYA) were negative for *P. perurans* at 25 dpc (Figure 3.5).

Figure 3.5: *Density (40 – Ct-value) of P. perurans in the challenge experiment at 11, 18 and 25 dpc (days post challenge). Green lines show the average Ct-value of P. perurans 11 (15.1), 18 (13.3) and 25 (15.6) dpc.*

3.2 Freshwater treatment: Location LA

At Location LA, freshwater treatment against AGD was conducted on September 18th, 2021. The site is located in Vestland County and was diagnosed with gill disease with the detection of *P. perurans.* Samples of the fish were collected before (15.09.21) and after (29.09.21) treatment. The average sea temperature one month before the treatment was 16.2 ± 1.1 °C, and the sea temperature the day of treatment was 14.8 °C. The fish were treated for 4 hours with freshwater at 14 °C. At the first sampling, the average weight and length of the fish was 1904.6 ± 615 grams and 53 ± 7 cm, respectively. The mean number of mobile lice the day before treatment was 0.35. The mean number of mobile lice the day after treatment was 0.05 (Figure 3.6).
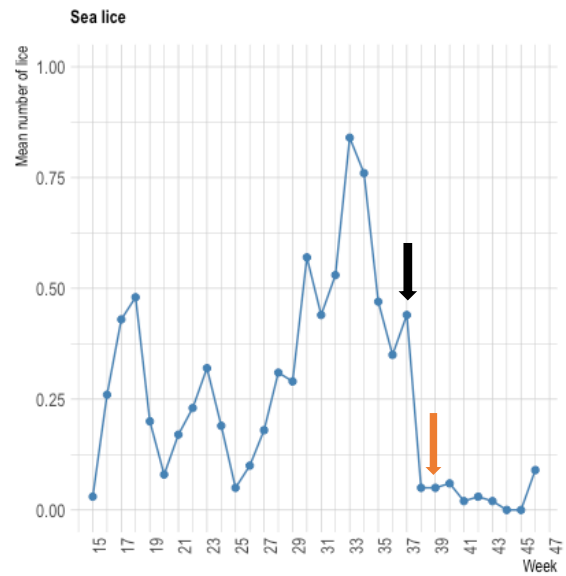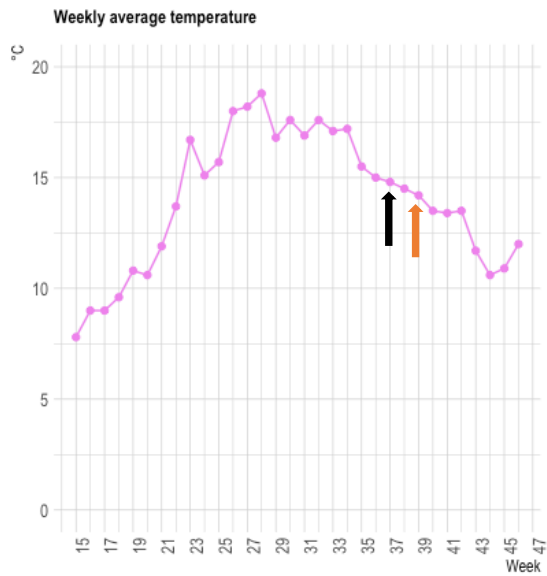
Figure 3.6: *Temperature in degrees Celsius (pink line) and mean number of sea lice (blue line) at Location LA from week 15 to 46 in 2021. The freshwater treatment and first sampling were conducted in week 37 (black arrows) and the sampling after freshwater treatment was conducted in week 39 (orange arrows). Data obtained from Barentswatch.no.*

All 30 salmon sampled before treatment had typical AGD lesions. Two of them had pale gills and one fish had snout injuries (Figure 3.8). The 30 salmon sampled after treatment had little visible external pathology, except for one fish with some mechanical damage (Figure 3.7). Findings from the 20 dead fish were pale gills in three of the fish and one fish had liver granulomas (Figure 3.8).
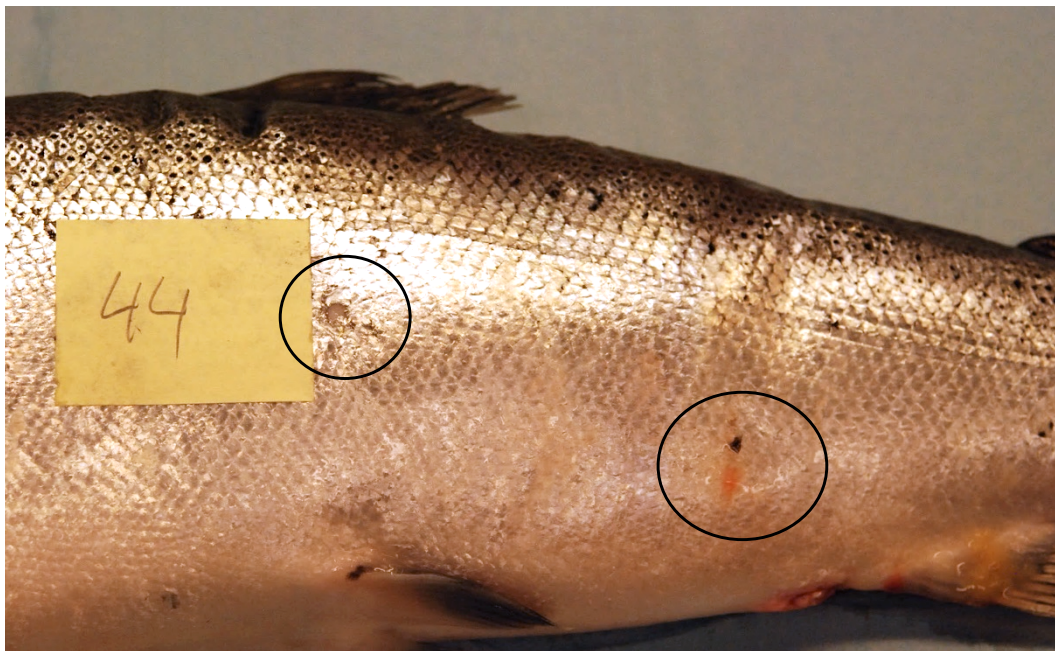

Figure 3.7: *Fish no. 44 (after treatment) with mechanical damage (black circles).*
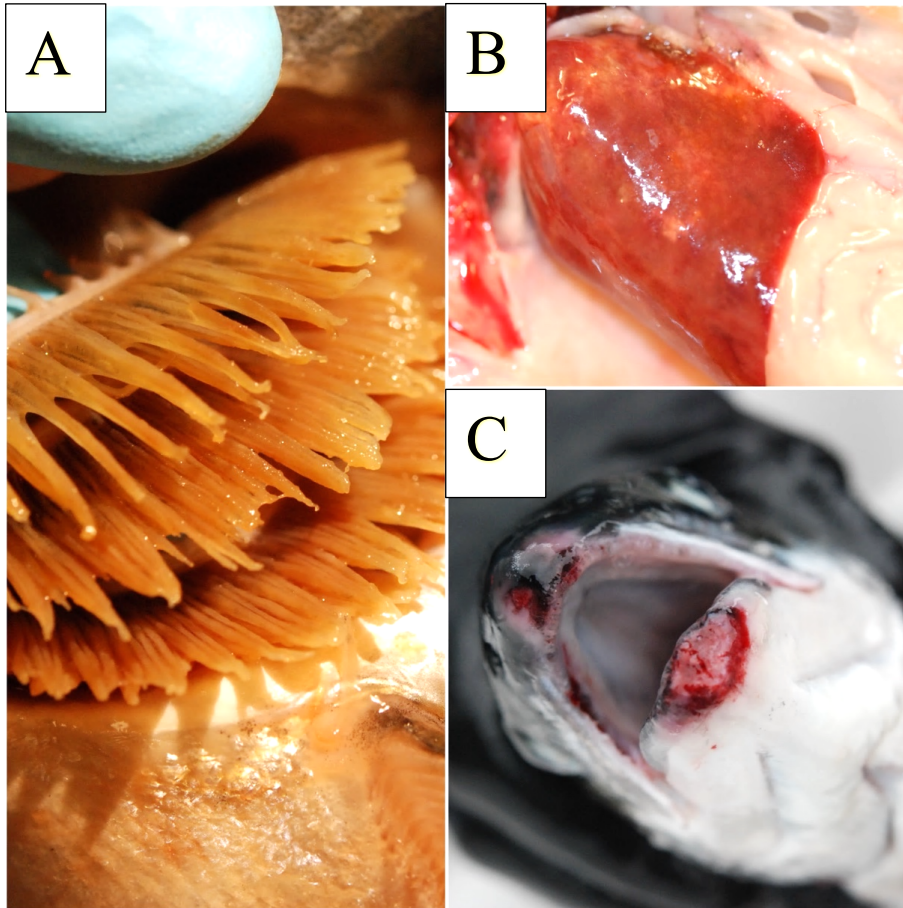
Figure 3.8: *A: Fish no. 33 (dead fish) with pale gills. B: Fish no. 37 (dead fish) with liver granulomas. C: Fish no. 20 (before treatment) with snout injuries.*

The total daily mortality in the sea cages the last 14 days before treatment varied between 30 and around 300 fish, while the total mortality during treatment and the three following days varied between 60 and 200 individuals (Figure 3.9). There was not a significant difference in mortality before vs. after freshwater treatment (p = 0.241). The mortality of the fish was 193 individuals the day after treatment.
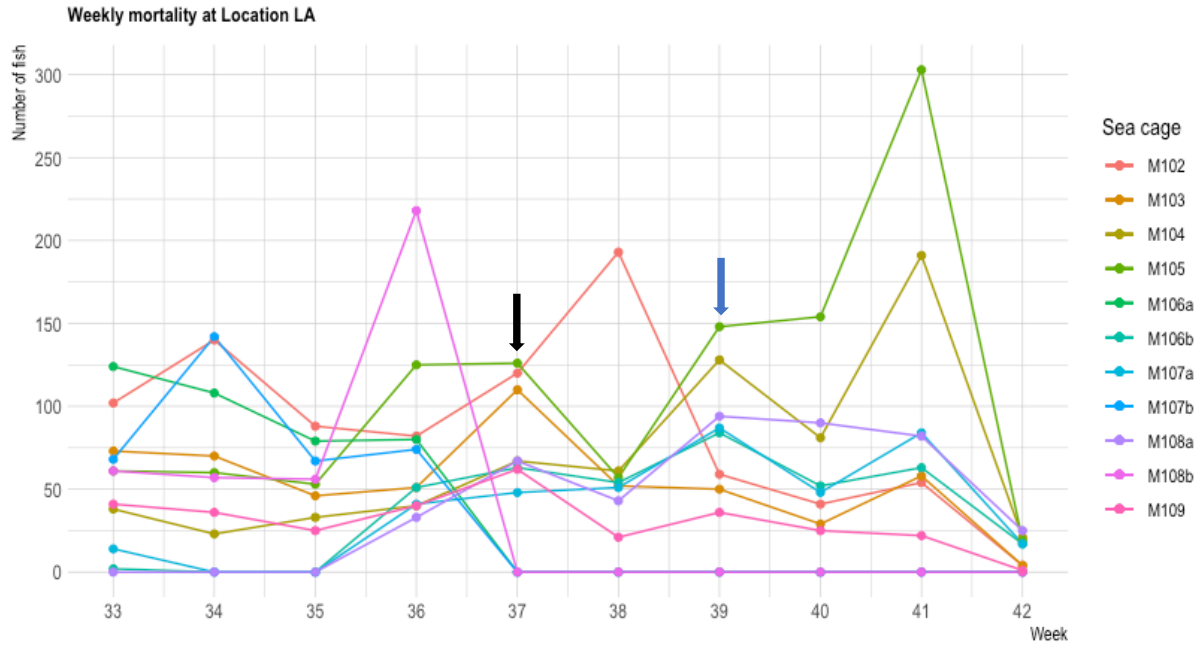
Figure 3.9: *Weekly mortality registered in the sea cages at Location LA from week 33 to 42 in 2021. Black arrow shows time of first sampling (15.09.21) and time of treatment (18.09.21). Blue arrow shows the time sampling after the freshwater treatment (29.09.21).*

Three delousing treatments were carried out in advance of this study at Location LA (one chemical treatment through feed and two mechanical treatments). The chemical delousing treatment was given through pellets containing Teflubenzuron (Ektobann vet. Skretting) in week 19. The mechanical treatments against sea lice were conducted in week 25 and 35. The mean number of adult female louse, mobile and fixed stages of louse are shown in Figure 3.10. The mean louse number before treatment was 0.146, while the mean louse number after treatment was 0.017. Fish with louse induced injuries were registered (Figure 3.11).
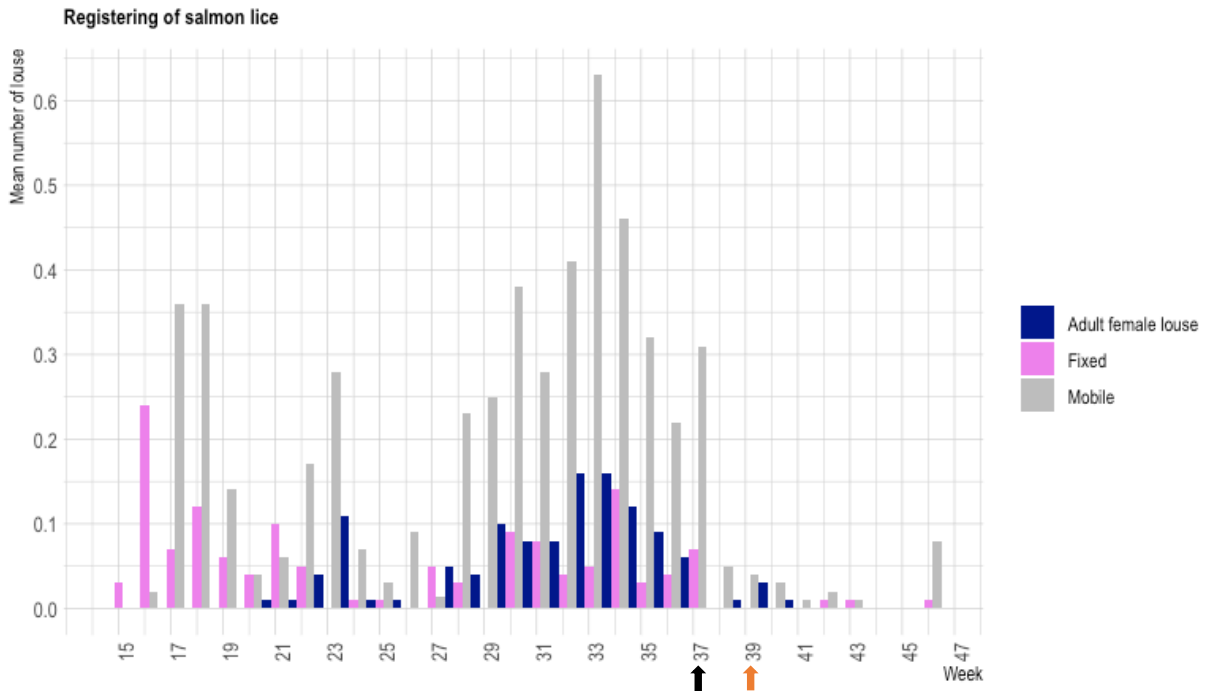
Figure 3.10: *Overview of the registered salmon lice at Location LA between week 15 and 46 in 2021. Black arrow = sampling of fish before freshwater treatment against AGD, orange arrow = sampling of fish after treatment. Freshwater treatment was conducted in week 37.*

Figure 3.11*: Fish no. 1 (before treatment) with louse induced injuries on its ventral surface.*

3.2.1 Gill score

Fish with lice induced injuries were registered and all the fish from the sampling before treatment had relatively high gill scores (Figure 3.12). Gill score for all the gill arches on the left side of the fish (8 surfaces) was registered.
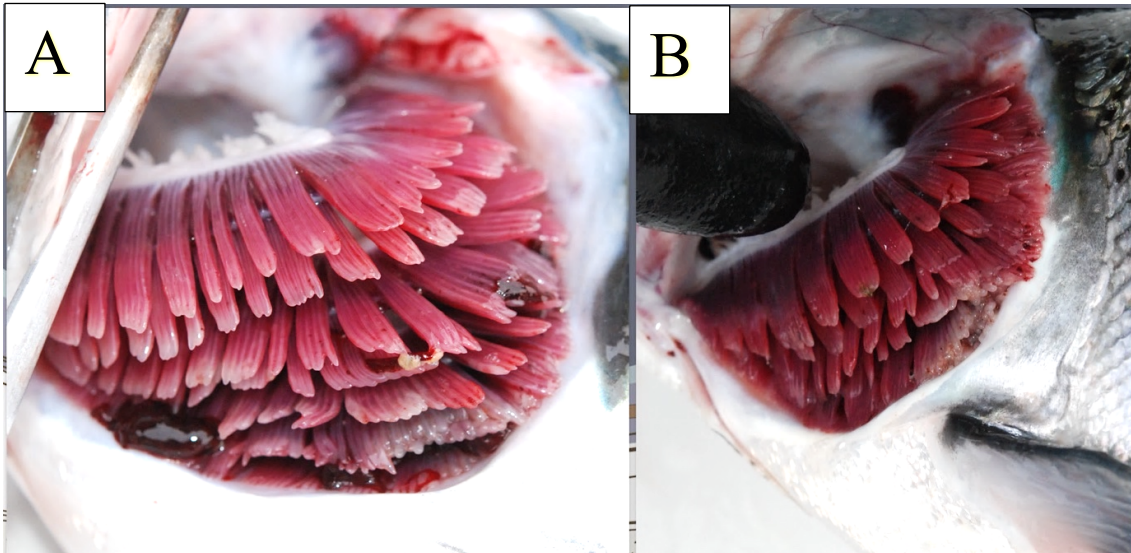
Figure 3.12: *A & B: Fish no. 10 (before treatment) with gill pathology.*

Figure 3.13 shows the average gill score of each individual fish before and after freshwater treatment. The average gill 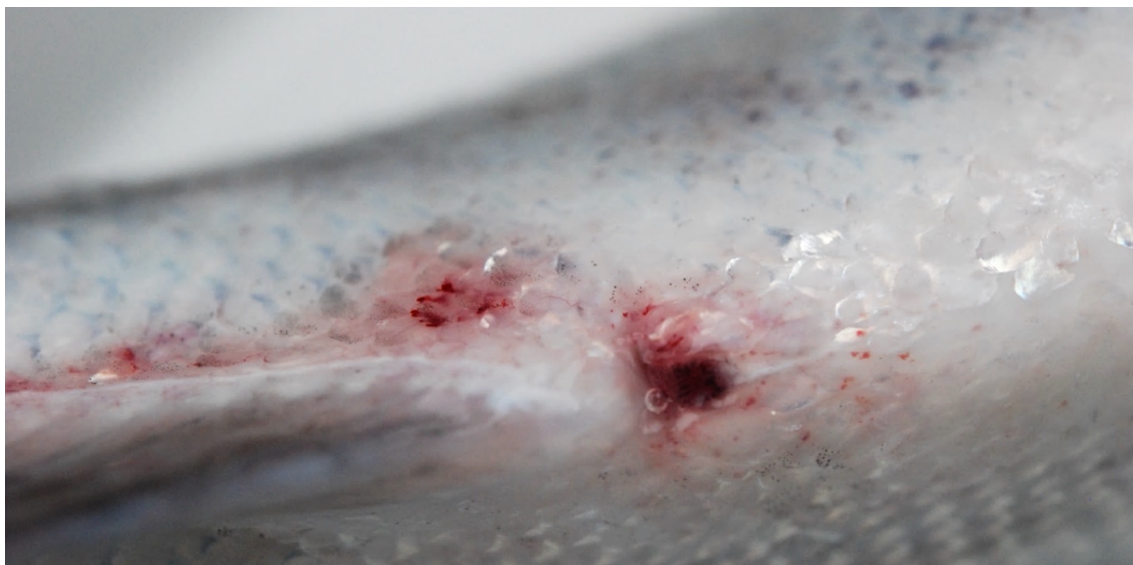score was significantly reduced after treatment compared to before treatment ($p \leq 0.0001$). The average gill score before and after treatment was 2.4 and 0.35, respectively. The highest average individual gill score registered before treatment was 3.4 ± 0.5, while the highest average individual gill score after treatment was 2.6 ± 0.35 (Figure 3.13).

Figure 3.13: *Average gill score of each individual fish at Location LA before (blue) and after (grey) freshwater treatment. Straight lines indicate the average gill score, and the dotted lines show the highest and lowest average individual gill score. Fish No. 1-30: Before treatment. Fish No. 51-80: After treatment.*

3.2.2 Microparasites

The samples (gills and kidney) were analysed for a selection of microparasites (Table 3.3). The prevalence of Piscine orthoreovirus 1 (PRV1), *P. theridion, P. perurans*, *Cand.* Syngnamydia salmonis, *Cand.* Piscichlamydia 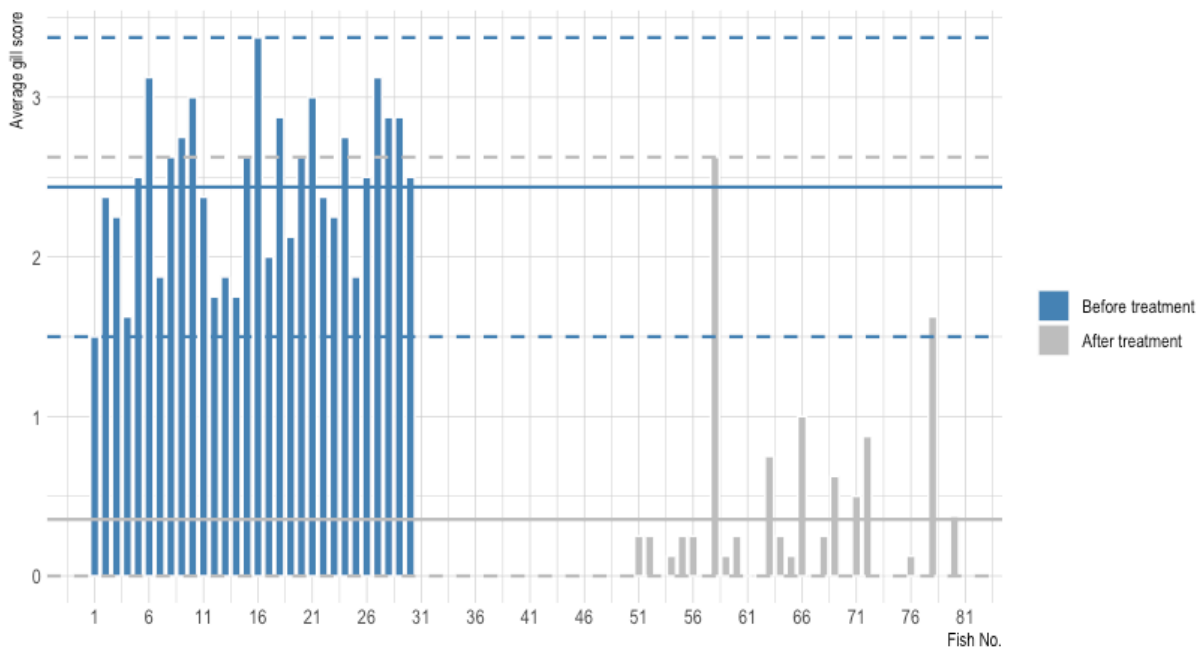salmonis and *Cand.* Branchiomonas cysticola on the gills was approximately 100 % in all sampling groups. All the fish were negative for Salmonid alphavirus (SAV), Infectious pancreas necrosis virus (IPNV), *Salmoxcellia vastator* and *Tetracapsuloides bryosalmonae* on the gills, and only a few fish were positive for Infectious salmon anemia virus HPR0 (ISAV HPR0), Piscine myocarditis virus (PMCV) and *Parvicapsula pseudobranchicola*. The prevalence of *Ichthyobodo* spp. and *Tenacibaculum* spp. was reduced after the freshwater treatment, while the prevalence increased for Salmon gill poxvirus (SGPV).

Table 3.3: Prevalence given in percent and number of positive individuals for a selection of microparasites on the gills of salmon at Location LA before and after freshwater treatment and in the dead fish. SAV = Salmonid Alphavirus, ISAV = Infectious salmon anemia virus, PRV1 = Piscine orthoreovirus 1, PMCV = Piscine myocarditis virus, SGPV = Salmon gill poxvirus, IPNV = Infectious pancreas necrosis virus, P. theridion = Paranucleospora theridion, P. perurans = Paramoeba perurans, Costia = Ichthyobodo spp., PKX = Tetracapsuloides bryosalmonae, Parvi = Parvicapsula pseudobranchicola, Ca. B. c. = Candidatus Branchiomonas cysticola, Ca. S. s. = Candidatus Syngnamydia salmonis, Ca. P. s. = Candidatus Piscichlamydia salmonis, TB-tuf = Tenacibaculum spp. N = 30 fish before treatment, N = 20 dead fish, N = 30 fish after treatment.

	Before treatment		Dead fish		After treatment	
	Number of positive individuals	Prevalence (%)	Number of positive individuals	Prevalence (%)	Number of positive individuals	Prevalence (%)
SAV	0/30	0	0/20	0	0/30	0
ISAV HRP0	4/30	13	1/20	5	0/30	0
PRV1	30/30	100	20/20	100	30/30	100
PMCV	0/30	0	0/20	0	1/30	3.3
SGPV	14/30	46.7	18/20	90	30/30	100
IPNV	0/30	0	0/20	0	0/30	0
P. theridion	30/30	100	20/20	100	30/30	100
P. perurans	30/30	100	19/20	95	29/30	96.7
Costia	30/30	100	2/20	10	6/30	20
S. vastator	0/30	0	0/20	0	0/30	0
PKX	0/30	0	0/20	0	0/30	0
Parvi	2/30	6.7	0/20	0	0/30	0
Ca. B. c.	30/30	100	20/20	100	30/30	100
Ca. S. s.	30/30	100	20/20	100	27/30	90
Ca. P. s.	30/30	100	20/20	100	30/30	100
TB-tuf	19/30	63.3	15/20	75	9/30	30

The density of a selection of pathogens is presented below as normalized expression (NE) values and reversed Ct-values (40 – Ct-value).

The prevalence of *P. perurans* on the gills was approximately 100 % in all sampling groups. The average density (40 – Ct-value) of *P. perurans* was 25.7 ± 2.1 before treatment, while the average density was

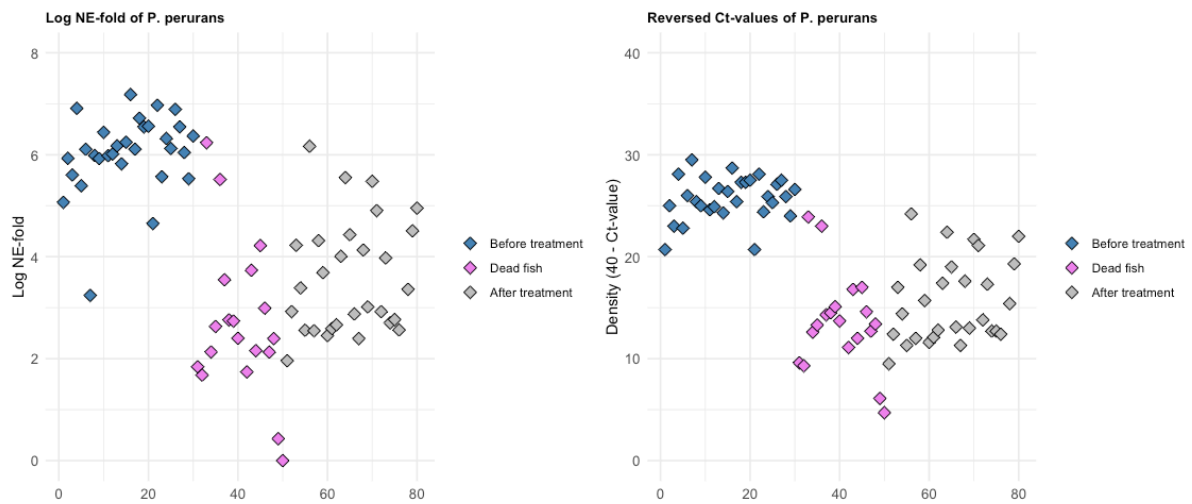15.7 ± 4 after treatment. The average density of *P. perurans* in the dead fish was 13.6 ± 4.7 (Figure 3.14).

Figure 3.14: *Density of P. perurans on the gills of salmon before (blue dots) and after (grey dots) freshwater treatment, and in the dead fish (pink dots) at Location LA. The data are represented as Log NE-fold and reversed Ct-values (40 – Ct-value). N = 30 positive individuals before treatment, N = 29 positive individuals after treatment, and N = 19 positive individuals of the dead fish.*

The prevalence of *Ichthyobodo* spp. was reduced from 100 % before treatment to 20 % after treatment. The prevalence of *Ichthyobodo* spp. was 10 % in the dead fish. The average density of *Ichthyobodo* spp. was 14.5 ± 1.7 before treatment, 13.4 ± 2 after treatment, and 18.6 ± 2.2 in the dead fish (Figure 3.15).
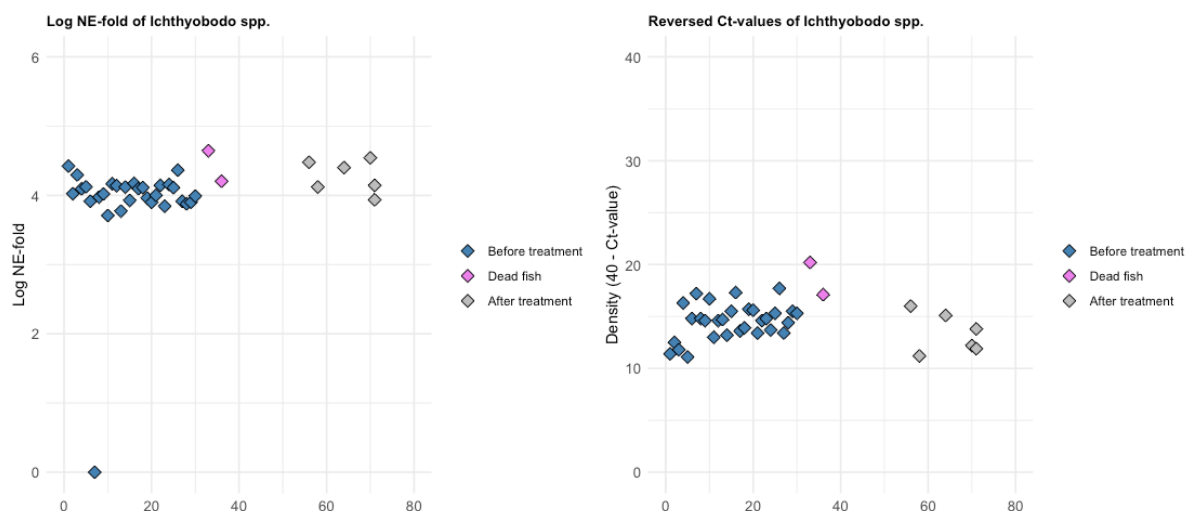

Figure 3.15: *Density of Ichthyobodo spp. on the gills of salmon before (blue dots) and after (grey dots) treatment, and in the dead fish (pink dots) at Location LA. The data are represented as Log NE-fold and reversed Ct-values (40 – Ct-value). N = 30 positive individuals before treatment, N = 2 positive individuals of the dead fish, and N = 6 positive individuals after treatment.*

The prevalence of *Cand.* Syngnamydia salmonis was 100 % on the gills before treatment and in the dead fish. The prevalence on the fish after treatment was 90 %. The average density of *Cand.* Syngnamydia salmonis was 17.6 ± 2.1 in the group before treatment, while the average density in the group after treatment was 8.4 ± 4.4. The average density of *Cand.* Syngnamydia salmonis in the dead fish was 12.5 ± 3.6 (Figure 3.16).

Figure 3.16: *Density of Candidatus Syngnamydia salmonis on the gills of salmon before (blue dots) and after (grey dots) treatment, and in the dead fish (pink dots) at Location LA. The data are represented as Log NE-fold and reversed Ct-values (40 – Ct-value). N = 30 positive individuals before treatment, N = 20 positive individuals of the dead fish and N = 28 positive individuals after treatment.*

The prevalence of *Cand.* Branchiomonas cysticola was 100 % in all the analysed gill tissue in all three sampling groups. The average density before treatment was 20.1 ± 1.8, while the average density after treatment was 21.9 ± 3. The average density of *Cand.* Branchiomonas cysticola in the dead fish was 21.9 ± 2 (Figure 3.17).

Figure 3.17: Density of Candidatus Branchiomonas cysticola on the gills of salmon before (blue dots) and after (grey dots) treatment, and in the dead fish (pink dots) at Location LA. The data are represented as Log NE-fold and reversed Ct-values (40 – Ct-value). N = 30 positive individuals before treatment, N = 20 positive individuals of the dead fish, and N = 29 positive individuals after treatment.

The prevalence of *Cand.* Piscichlamydia salmonis was 100 % in all the analysed gill tissue at all three sampling groups. The average density of *Cand.* Piscichlamydia salmonis was 16.4 ± 3.1 before treatment and 16.6 ± 3.2 after treatment. The average density of *Cand.* Piscichlamydia salmonis in the dead fish was 20.5 ± 3.1 (Figure 3.18).
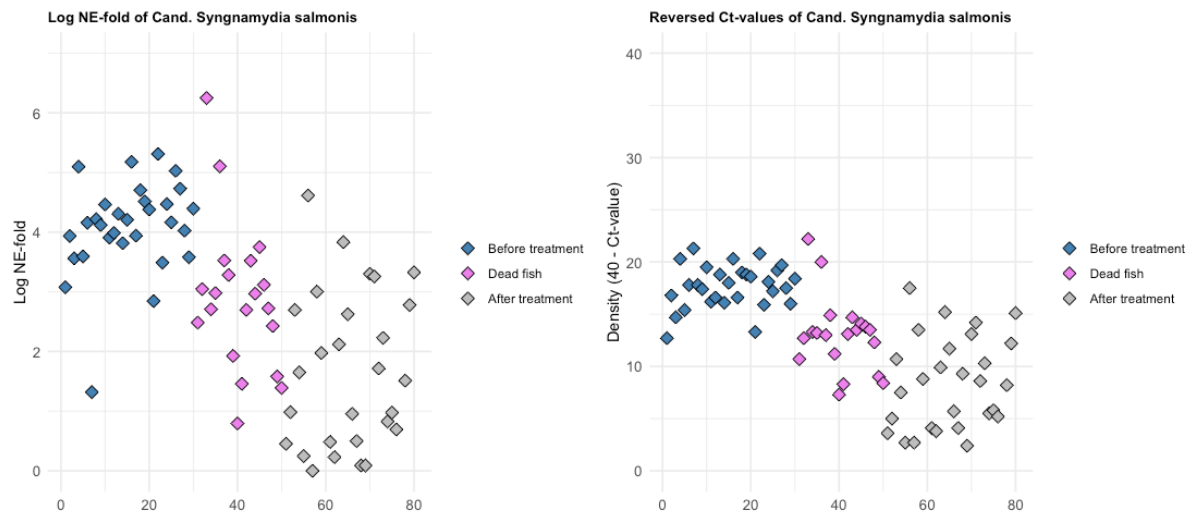

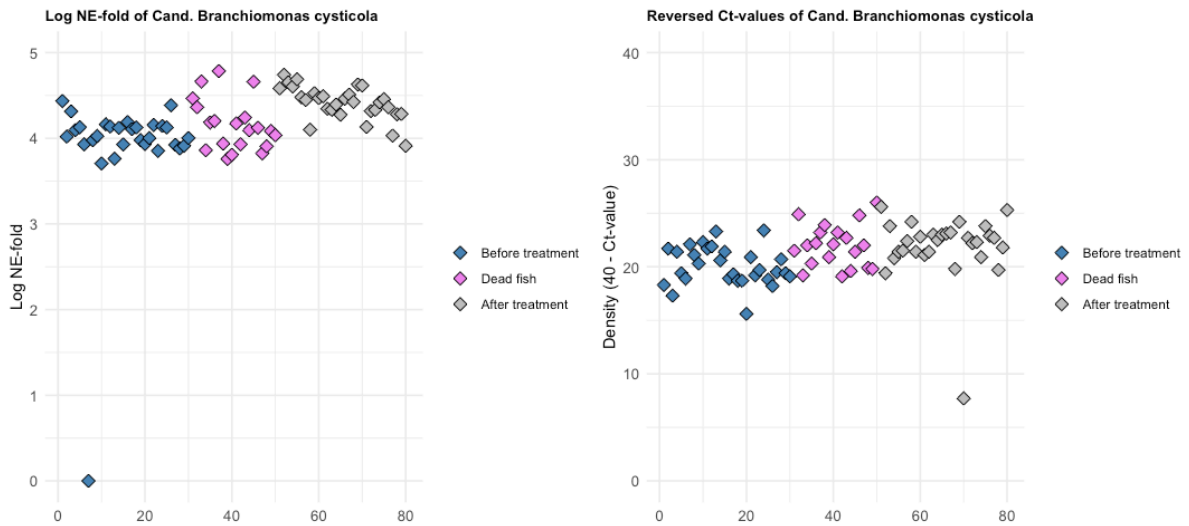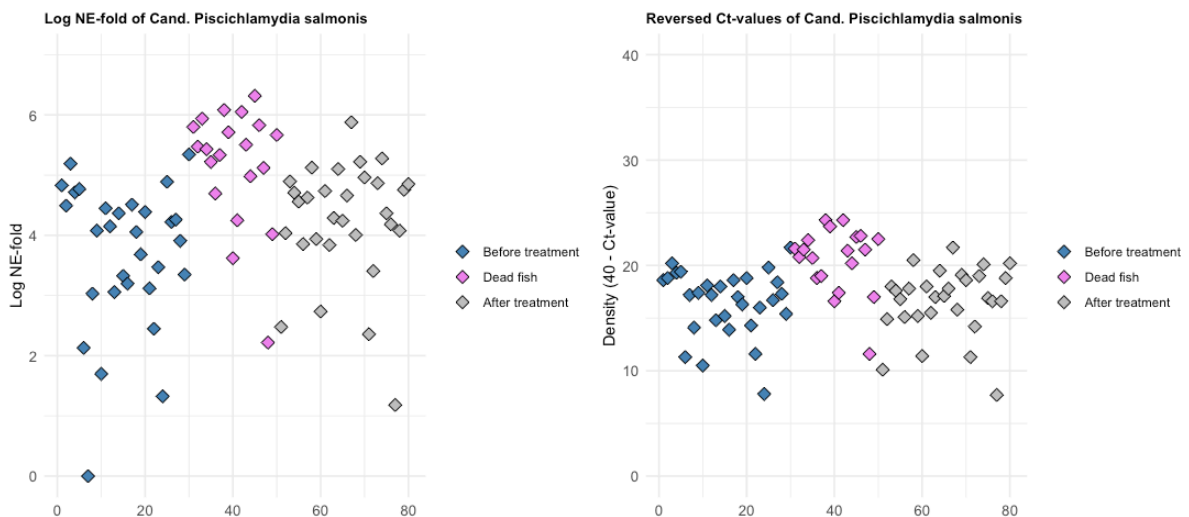*Figure 3.18: Density of Candidatus Piscichlamydia salmonis on the gills of salmon before (blue dots) and after (grey dots) treatment, and in the dead fish (pink dots) at Location LA. The data are represented as Log NE-fold and reversed Ct-values (40 – Ct-value). N = 30 positive individuals before treatment, N = 20 positive individuals of the dead fish, and N = 30 positive individuals after treatment.*

All fish from the three sampling groups were positive for Piscine orthoreovirus 1 (PRV1) on the gills. The prevalence was 100 % before and after treatment, and 100 % in the dead fish. The average density of PRV1 was 16.9 ± 1.5 before treatment and 18.2 ± 1.9 after treatment. The average density of PRV1 was 13.5 ± 1.5 in the dead fish (Figure 3.19).
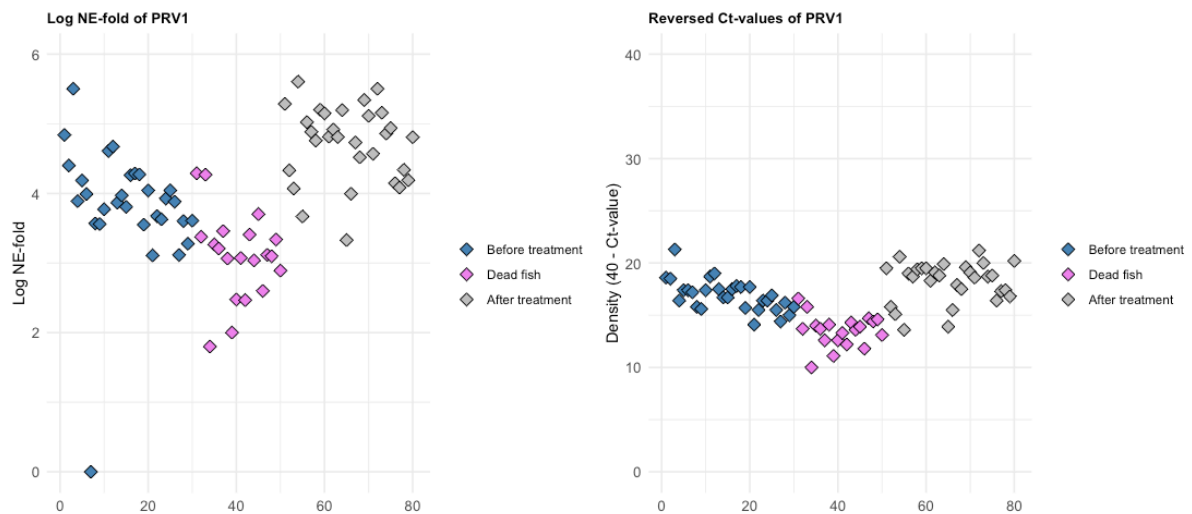
Figure 3.19: *Density of Piscine orthoreovirus 1 (PRV1) on the gills of salmon before (blue dots) and after (grey dots) freshwater treatment, and in the dead fish (pink dots) at Location LA. The data are represented as Log NE-fold and reversed Ct-values (40 – Ct-value). N = 30 positive individuals before treatment, N = 30 positive individuals after treatment, and N = 20 positive individuals of the dead fish.*

The prevalence of Salmon gill poxvirus (SGPV) on the gills increased from 46.7 % before treatment to 100 % after treatment. The prevalence of SGPV in the dead fish was 90 %. The average density of SGPV before treatment was 10.2 ± 5.8, while the average density after treatment was 13.7 ± 2.6. The average density of SGPV in the dead fish was 10.8 ± 5.97 (Figure 3.20).

Figure 3.20: *Density of Salmon gill poxvirus (SGPV) on the gills of salmon from before (blue dots) and after (grey dots) freshwater treatment, and in the dead fish (pink dots) at Location LA. The data are represented as Log NE-fold and reversed Ct-values (40 – Ct-value). N = 14 positive individuals before treatment, N = 30 positive individuals after treatment, and N = 18 positive individuals of the dead fish.*

The prevalence of *P. theridion* on the gills was 100 % in all the fish from all three sampling groups. The average density of *P. theridion* before treatment was 24.9 ± 1.7, while the average density was 22.4 ± 2 after treatment. The average density of *P. theridion* in the dead fish was 27.6 ± 2.8 (Figure 3.21).

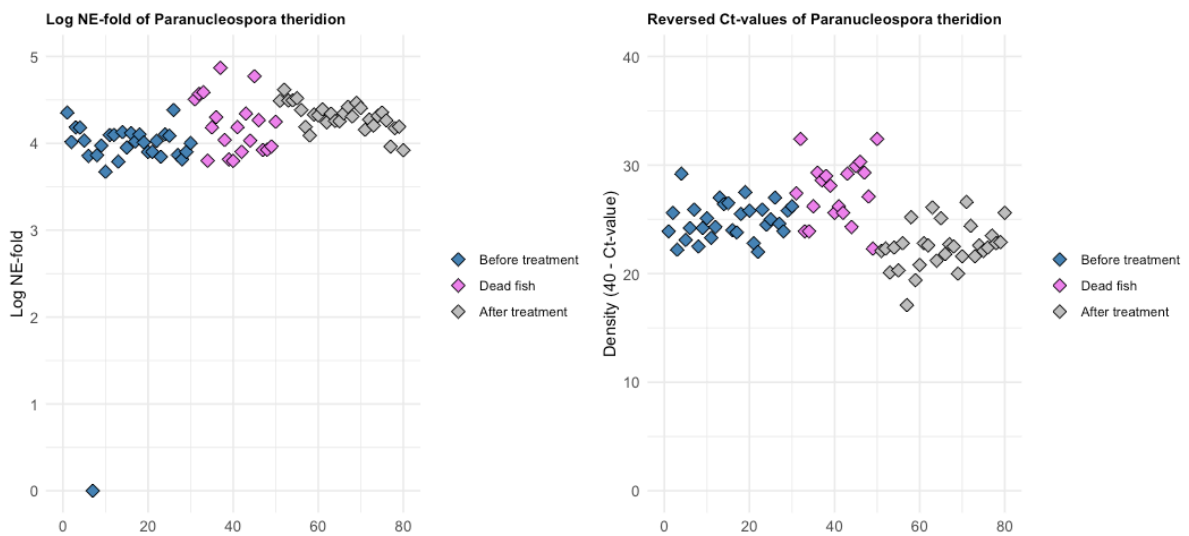***Figure 3.21:*** *Density of Paranucleospora theridion on the gills of salmon from before (blue dots) and after (grey dots) freshwater treatment, and in the dead fish (pink dots) at Location LA. The data are represented as Log NE-fold and reversed Ct-values (40 – Ct-value). N = 30 positive individuals before treatment, N = 30 positive individuals after treatment, and N = 20 positive individuals of the dead fish.*

In addition to the pathogens presented above, high Ct-values of *Tenacibaculum* spp. on the gills were detected.

The p-values of the analysed tissue samples are given in the appendix.

3.2.3 Histology

Histological sections of the gills show histological changes compatible with AGD lesions, such as clubbing of the secondary lamellae (Figure 3.25) and hyperplasia of mucus cells (Figure 3.26). The highest mean histological gill score before freshwater treatment was observed with lifting (parameter III) (Figure 3.27) and fresh aneurisms (parameter VII), whereas the highest histological mean gill score after the treatment was observed with thickened distal primary filament (parameter VI), lifting (parameter III), fresh aneurisms (parameter VII) and bleeding aneurisms (parameter VIII) (Figure 3.23). Multifocal fusions of the secondary lamellae forming caverns was also observed, containing amoeba-like cells (Figure 3.24). The most frequent parameter given before treatment was hyperplasia of mucus cells (parameter I) (Figure 3.26) and hypertrophy of epithelial cells (parameter IV) (Figure 3.26). After treatment, the most frequent parameter given was hyperplasia of mucus cells (parameter I). Most gill scores were given as 1 and 2, and few histological sections were scored as 3 (Figure 3.23).
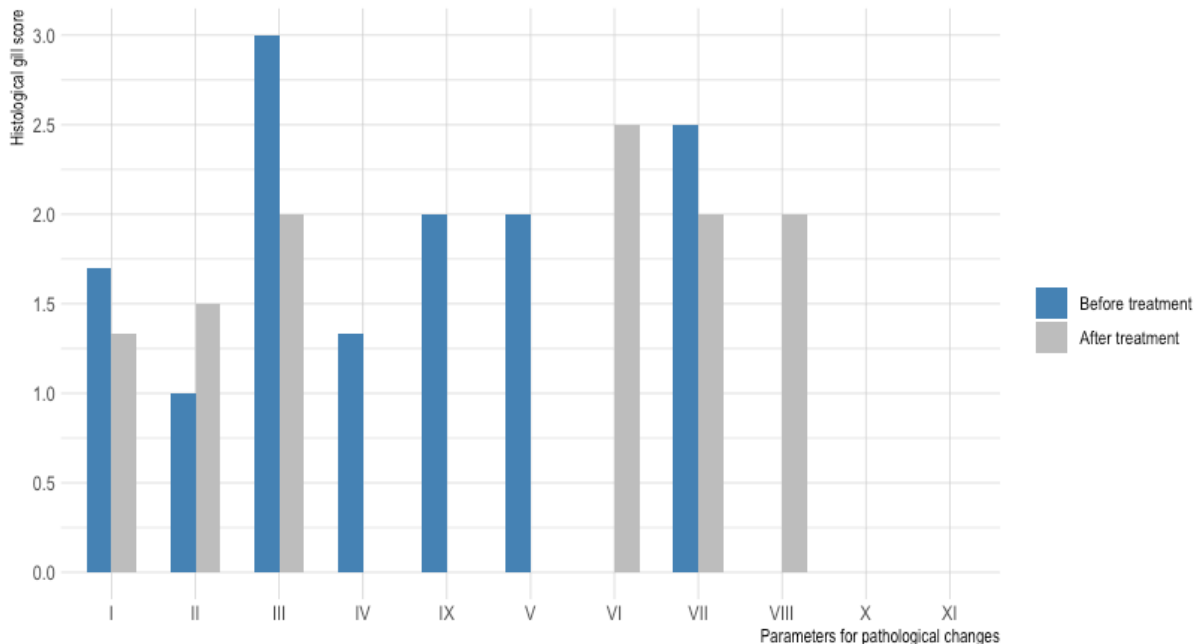
Figure 3.23: *Mean histological gill score of the fish before (blue) and after (grey) freshwater treatment at Location LA. Eleven pathological changes are included in this study of Atlantic salmon. **I:** Hyperplasia of mucus cells, **II:** Clubbing, **III:** Lifting, **IV:** Hypertrophy of epithelial cells, **V:** Hyperplasia of epithelial cells, **VI:** Thickened distal primary filament, **VII:** Fresh aneurisms, **VIII:** Bleeding aneurisms, **IX:** Old aneurisms, **X:** Inflammation, **XI:** Necrosis. N = 30 fish before treatment, N = 30 fish after treatment.*
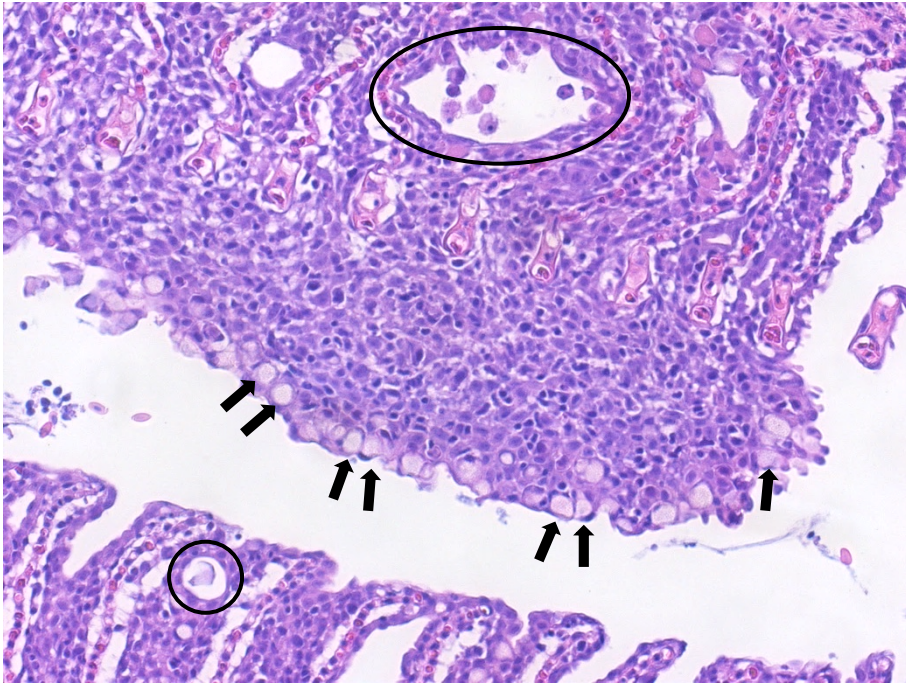
Figure 3.24: *Amoeba like cells in interlamellar vesicles (cavities) in fish before treatment at Location LA. The pathological changes are compatible with AGD lesions such as hyperplasia and hypertrophy of epithelial cells, caverns, inflammation, and hyperplasia of mucus cells (black arrows). Black circles show amoeba like cells in caverns. Scale: rod = 20 µm.*

Figure 3.25: *Fish before treatment at Location LA. Black circles show clubbing of secondary lamellae. Scale: rod = 20 µm.*
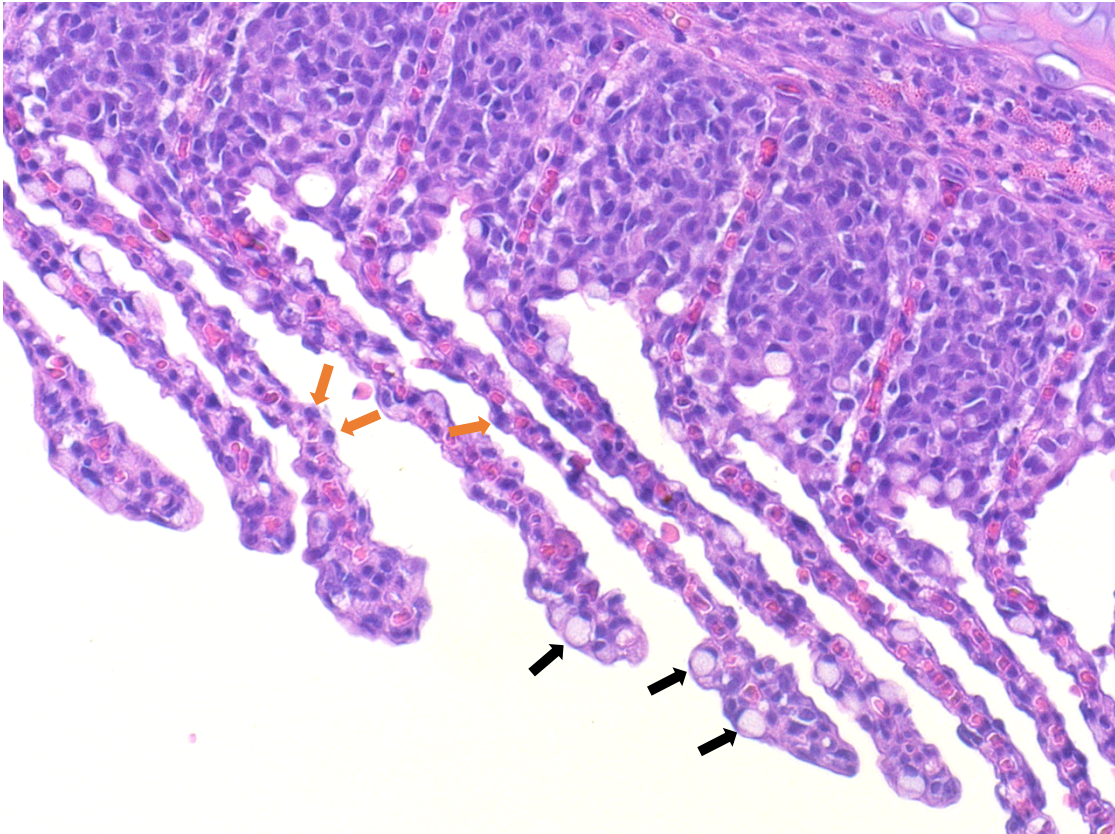
Figure 3.26: *Histological section of untreated fish from Location LA with pathological changes such as hyperplasia of mucus cells (black arrows) and hypertrophy of epithelial cells (orange arrows). Scale: rod = 20 µm.*
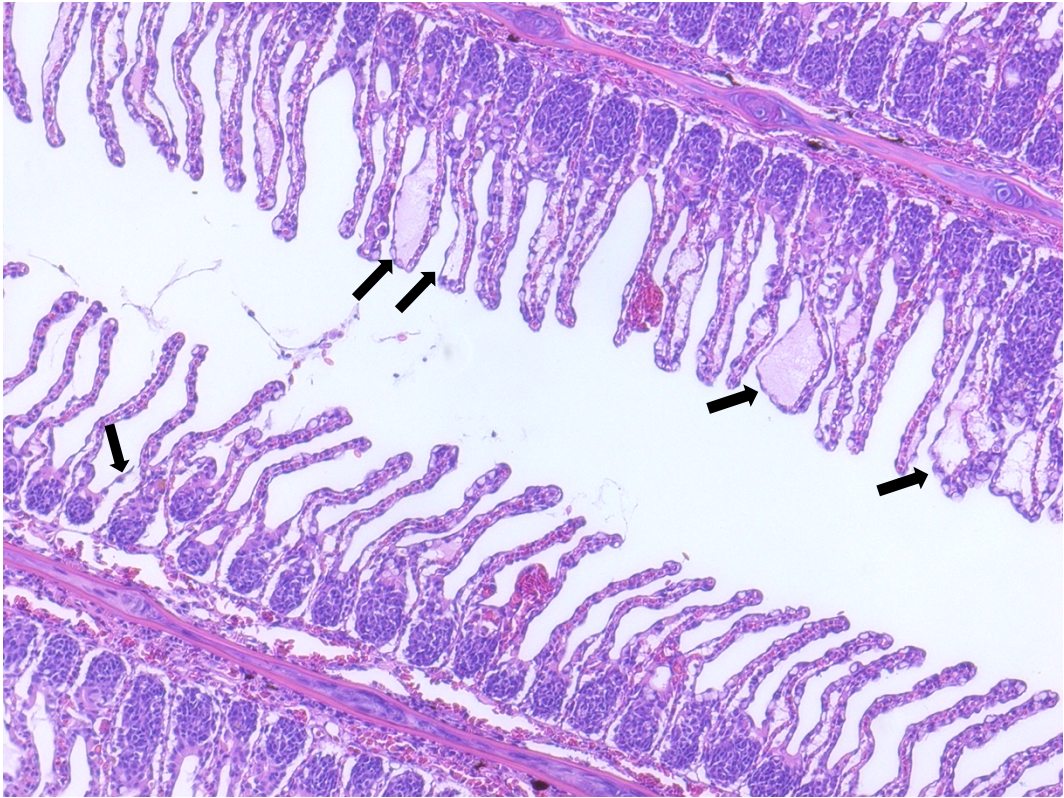
Figure 3.27: Untreated fish at Location LA with lifting. Scale: rod = 100 µm.

3.3 Cold freshwater and thermal delousing: Location LB

Location LB, in Vestland County, conducted a treatment using cooled freshwater followed by thermal delousing on September 6th, 2021. The average sea temperature one month before the treatment was 14.5 °C. The sea temperature the day of treatment was 14.7 °C. The fish were directly transferred to the freshwater at 8 °C. Among the 30 salmon sampled before treatment (06.09.21), the mean number of mobile lice was 8.6, while 11 days after treatment (17.09.21) the mean number of mobile lice was 0.67. On the dead fish (08.09.21), the mean number of mobile lice was 1.3 (Figure 3.28). At the first sampling, the average weight and length of the fish were 1099 ± 748 grams and 44.3 ± 9 cm, respectively. The fish started to eat the same day the treatment was conducted.
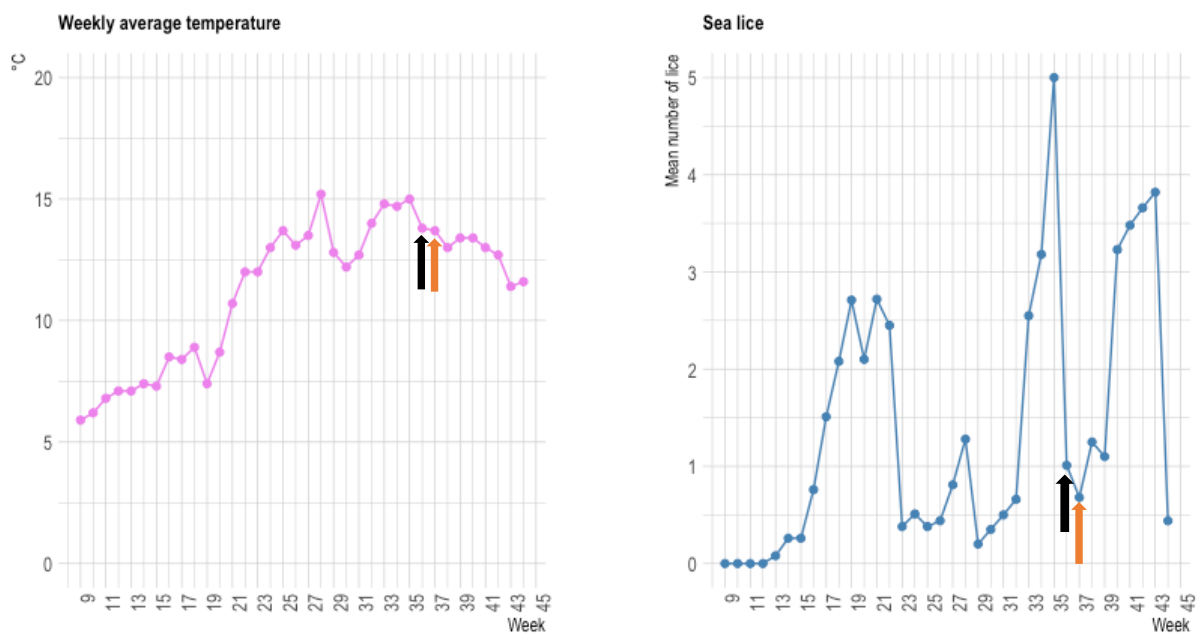
Figure 3.28: *Weekly temperature (pink) and mean number of sea lice (blue) at Location LB from week 9 to 44 in 2021. The treatment was conducted in week 36 (black arrows) and the sampling after treatment was done in week 37 (orange arrows). Data obtained from Barentswatch.no.*

Louse induced damage was observed before and after treatment, while gill changes was primarily observed before treatment (Figure 3.29). The changes (hyperplasia of the gill epithelia) were compatible with AGD lesions caused by *P. perurans* (Figure 3.29A), while other changes of the gills have previously been associates with *P. theridion* and SGPV (Figure 3.29B). After treatment, small petechial haemorrhages in the skin were detected in a few salmon (Figure 3.29D). Fish with louse induced injuries were registered before treatment (Figure 3.29C).
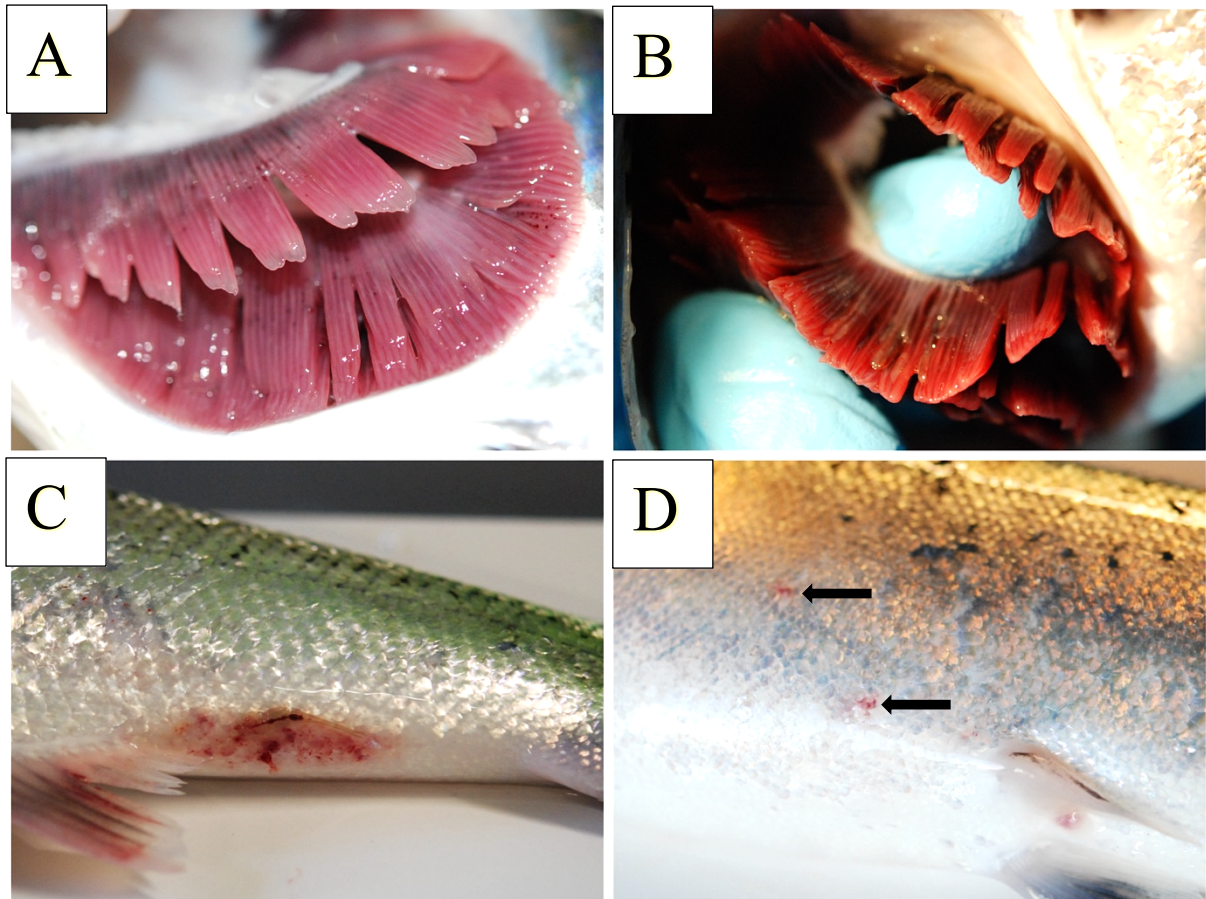
Figure 3.29: *Fish sampled from Location LB. A & B: Fish no. 9 (before treatment) with gill changes (A. epithelial cell hyperplasia and B. hyperplasia and necrosis). C: Fish no. 9 (before treatment) with louse induced injuries. D: Fish no. 88 (after treatment) with skin lesions (black arrows).*

The average mortality in the treated sea cage one month before treatment was 10.6 individuals each day (0.01 %). The average mortality was 41.6 individuals each day the two days after treatment. In the following days until the last sampling on September 17th, the average mortality was 11.8 individuals each day (0.01 %). There was not a significant increase in average mortality due to the treatment (p < 0.05) (Figure 3.30).
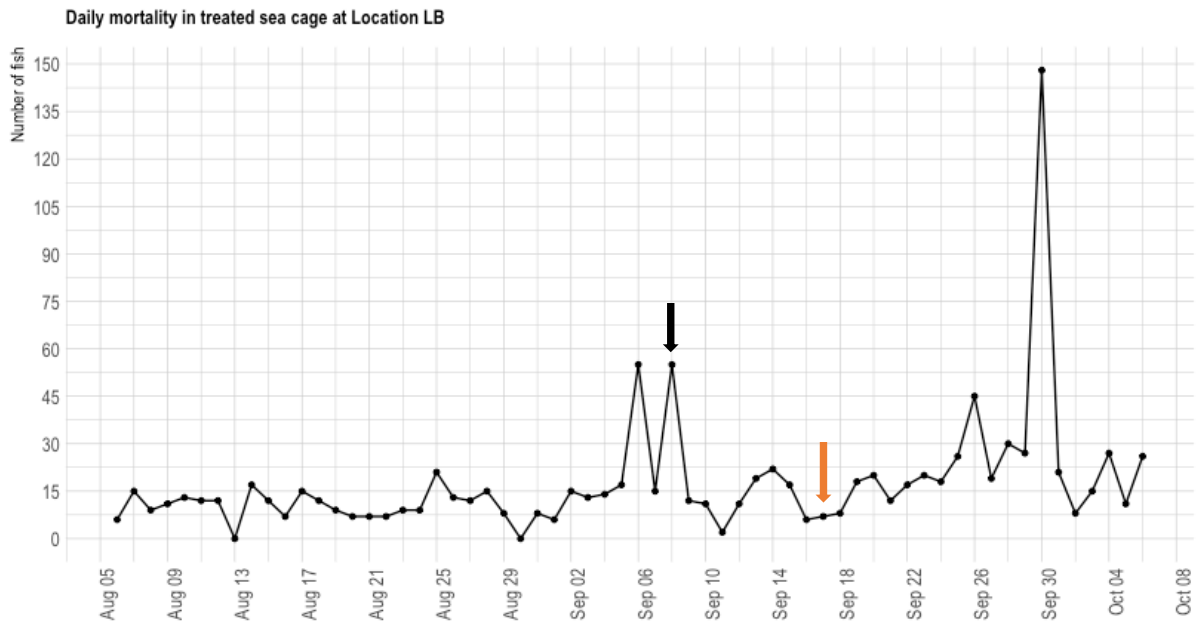
Figure 3.30: *Average daily mortality in treated sea cage at Location LB from August 6th to October 6th, 2021. The treatment was carried out September 8th (week 36). Black arrow = time of treatment. Orange arrow = time of sampling after treatment.*

The number of the ectoparasitic sea lice (*L. salmonis* and *C. elongatus*) was registered at all three samplings. 259 sea lice in total were registered before treatment, 40 lice on the dead fish, and 20 lice after treatment. Before treatment, 33 of the sea lice registered were *C. elongatus* females, while the remaining louse was *L. salmonis*. 140 female louse of *L. salmonis* were registered before treatment; 125 of them had egg strings and 15 of them were preadult females. 97 male lice of *L. salmonis* were registered (Figure 3.31). There was a significant reduction in the total number of lice between the groups "before treatment" and "dead fish" (p < 0.0001) and between the groups "before treatment" and "after treatment" (p < 0.0001). There was also a significant difference between the groups "dead fish" and "after treatment" (p = 0.038).
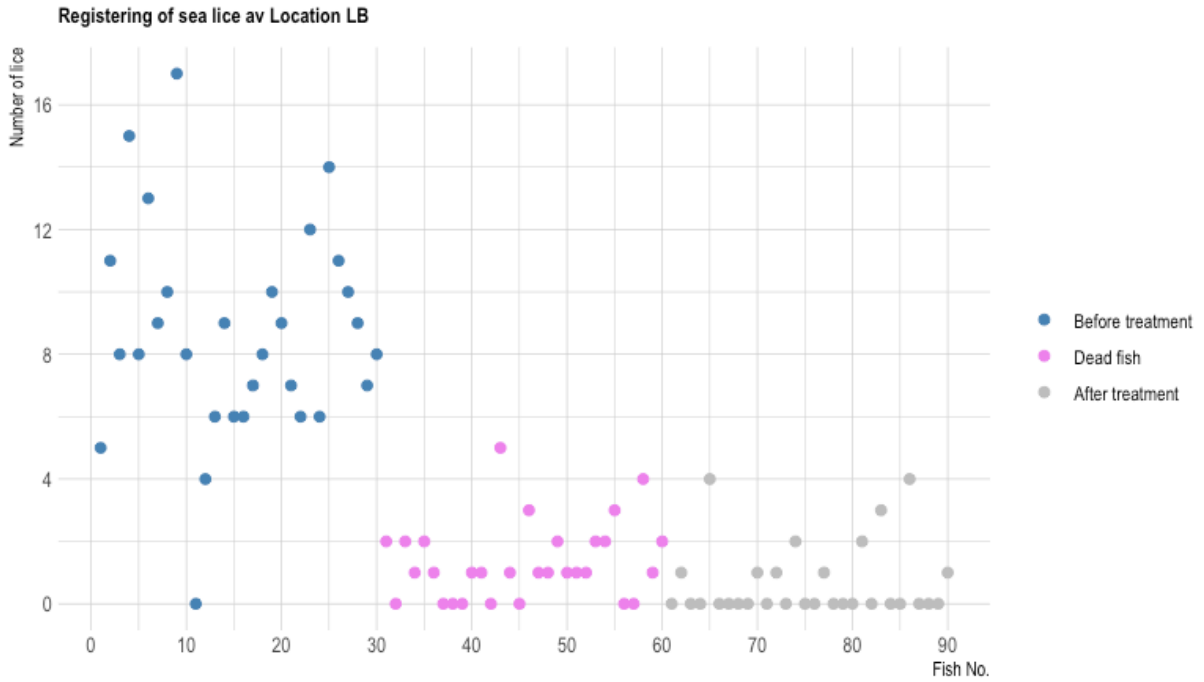
Figure 3.31: *Total number of sea lice before and after treatment and of the dead fish at Location LB. 259 sea lice in total were registered on the fish sampled before treatment (06.09.21), 40 sea lice on the dead fish (08.09.21), and 20 sea lice in total after treatment (17.09.21). Fish 1-30: Before treatment, fish 31-60: Dead fish, fish 61-90: After treatment.*

3.3.1 Gill score

Front and back of the left gill arches of the salmon were scored (Table 2.2). The average gill score before and after the treatment was 0.195 ± 0.25 and 0.204 ± 0.2, respectively. The highest gill score observed before and after treatment was score 2 (Figure 3.32). There was not a significant difference in the average gill score before compared to after treatment ($p = 0.84$).
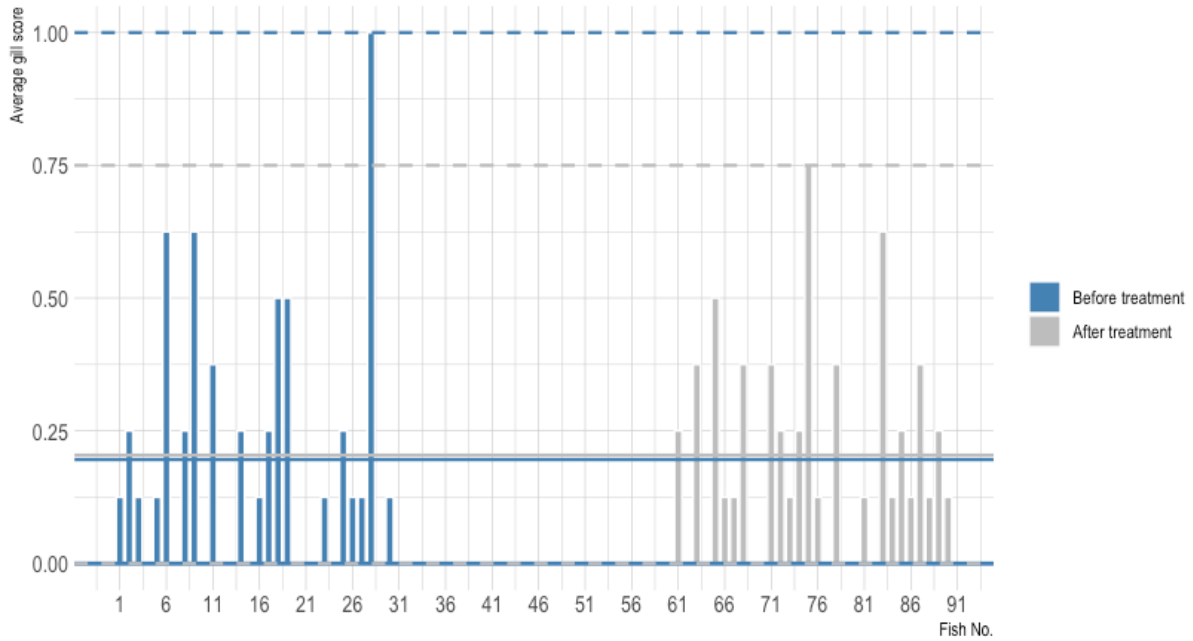
Figure 3.32: *The average gill score of each individual fish at Location LB before (blue) and after (grey) the treatment. Straight lines represent the average gill score, and the dotted lines show the highest and lowest average individual gill score. Fish No. 1-30 is before treatment. Fish No. 61-90 is after treatment.*

3.3.2 Elongation factor

The reference gene (EF1A) used as an internal control on the gills of Atlantic salmon in this study showed high Ct-values at Location LB in the group before treatment. EF1A on the gills from fish 8, 10, 11 and 14 differed from the other Ct-values of EF1A. The elongation factor for the gill tissue before treatment at Location LB were RNA-extracted three times and real-time RT-PCR were re-run six times. The average Ct-values for EL1A gill sample 8, 10, 11 and 14 from the first qPCR-round (old gill sample) and the last qPCR-round (new gill sample) are presented below in Table 3.5. Deviation in these EF1A-values will affect the normalized values presented further down in the results.

Table 3.5: *The average Ct-value for the first and last real-time RT-PCR rounds of the elongation factor for Atlantic salmon (EF1A) in the group before treatment at Location LB.*

Old gill sample 8	New gill sample 8	Old gill sample 10	New gill sample 10	Old gill sample 11	New gill sample 11	Old gill sample 14	New gill sample 14
22.2	22.8	18.9	18.0	27.1	18.9	20.3	20.9

3.3.3 Microparasites

The gills were analysed for the microparasites presented in Table 3.5. The prevalence of *Paranucleospora theridion* (Nuc), Piscine orthoreovirus 1 (PRV1) and *Candidatus* Branchiomonas cysticola (Ca. B. c.) was 100 % in all three sampling groups. The fish from all three samplings were negative for Salmonid Alphavirus (SAV), *Tetracapsuloides bryosalmonae* (PKX) and *Parvicapsula pseudobranchicola* (Parvi). Only a few fish in the groups before and after treatment were positive for

Infectious salmon anaemia virus (ISAV), Salmon gill poxvirus (SGPV) and Infectious pancreas disease virus (IPNV). The prevalence of *Ichthyobodo* spp. (Costia) increased from 36.7 % before treatment to 56.7 % after treatment, while the prevalence of *Candidatus* Syngnamydia salmonis (Ca. S. s.) was reduced from 70 % to 16.7 %. The prevalence of *P. perurans* was reduced from 100 % to 53.3 % (Table 3.5).

Table 3.6: Prevalence given in percent and number of positive individuals for a selection of microparasites on the gills of salmon at Location LB before and after treatment and in the dead fish. SAV = Salmonid Alphavirus, ISAV = Infectious salmon anemia virus, PRV1 = Piscine orthoreovirus 1, PMCV = Piscine myocarditis virus, SGPV = Salmon gill poxvirus, IPNV = Infectious pancreas necrosis virus, P. theridion = Paranucleospora theridion, P. perurans = Paramoeba perurans, Costia = Ichthyobodo spp., Yersinia = Yersinia ruckeri, PKX = Tetracapsuloides bryosalmonae, Parvi = Parvicapsula pseudobranchicola, Ca. B. c. = Candidatus Branchiomonas cysticola, Ca. S. s. = Candidatus Syngnamydia salmonis, Ca. P. s. = Candidatus Piscichlamydia salmonis, TB-tuf = Tenacibaculum spp.

	Before treatment		Dead fish		After treatment	
	Number of positive individuals	Prevalence (%)	Number of positive individuals	Prevalence (%)	Number of positive individuals	Prevalence (%)
SAV	0/30	0	0/30	0	0/30	0
ISAV	8/30	26.7	28/30	93.3	3/30	10
PRV1	30/30	100	30/30	100	30/30	100
PMCV	0/30	0	2/30	6.7	0/30	0
SGPV	3/30	10	21/30	70	4/30	13.3
IPNV	8/30	26.7	10/30	33.3	3/30	10
P. theridion	30/30	100	30/30	100	30/30	100
P. perurans	30/30	100	17/30	56.7	16/30	53.3
Costia	11/30	36.7	24/30	80	17/30	56.7
Yersinia	1/30	3	1/30	3	1/30	3
PKX	0/30	0	0/30	0	0/30	0
Parvi	0/30	0	0/30	0	0/30	0
Ca. B. c.	30/30	100	30/30	100	30/30	100
Ca. S. s.	21/30	70	22/30	73.3	5/30	16.7
Ca. P. s.	0/30	0	2/30	6.7	0/30	0
TB-tuf	3/30	10	16/30	53.3	5/30	16.7

The density of a selection of pathogens is presented below as normalized expression (NE) values and reversed Ct-values (40 – Ct-value).

The prevalence of *P. perurans* on the gills was 100 % before the treatment, whereas the prevalence in the dead fish and the group after treatment was 56.7 % and 53.3 %, respectively. The density (40 – Ct-value) of *P. perurans* before treatment was 12.8 ± 5.2, while the density was 10.0 ± 8.7 after treatment. The density of *P. perurans* in the dead fish was 11.1 ± 4.8 (Figure 3.33).
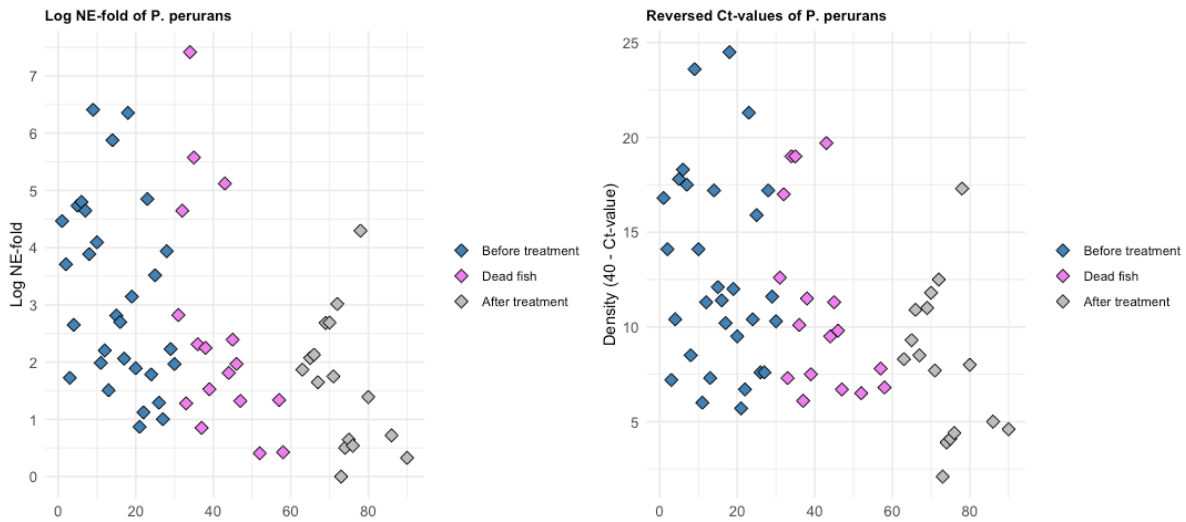
Figure 3.33: *Density of Paramoeba perurans on the gills of salmon from before (blue) and after (grey) treatment, and in the dead fish (pink dots) at Location LB. The data are represented as Log NE-fold and reversed Ct-values (40 – Ct-value). N = 30 positive individuals before treatment, N = 16 positive individuals after treatment, and N = 17 positive individuals of the dead fish.*

The prevalence of *Ichthyobodo* spp. was 36.7 % in the group before treatment, while the prevalence was 80 % in the dead fish. After the treatment the prevalence of *Ichthyobodo* spp. was 56.7 %. The density of *Ichthyobodo* spp. was 9.6 ± 4 before treatment, 8.3 ± 4.7 after treatment and 11.4 ± 6.7 in the dead fish (Figure 3.34).
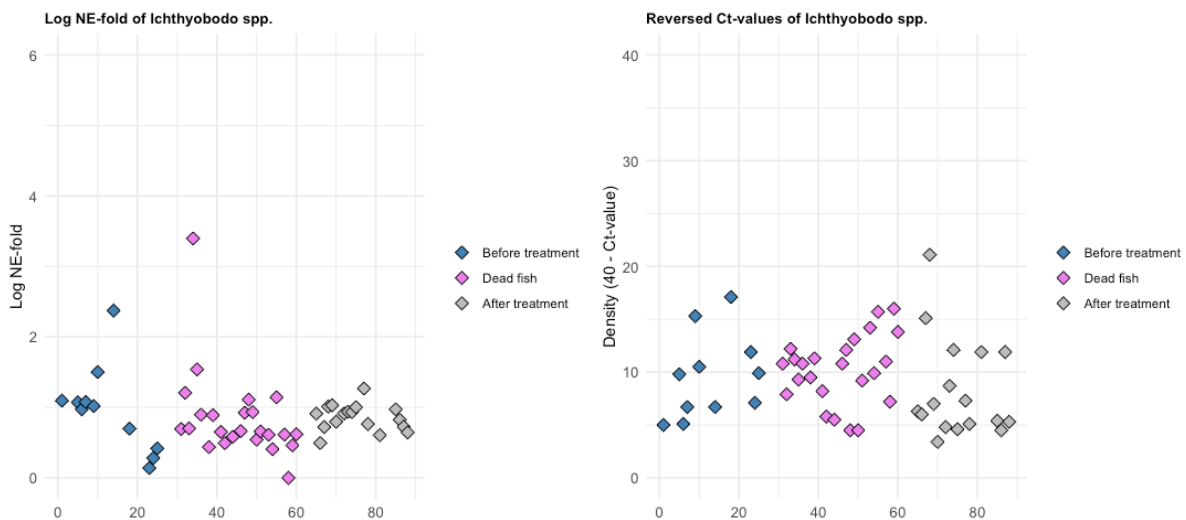
Figure 3.34: *Density of Ichthyobodo spp. on the gills of salmon from before (blue dots) and after (grey dots) treatment, and in the dead fish (pink dots) at Location LB. The data are represented as Log NE-fold and reversed Ct-values (40 – Ct-value). N = 11 positive individuals before treatment, N = 17 positive individuals after treatment, and N = 24 positive individuals of the dead fish.*

The prevalence of *Candidatus* Syngnamydia salmonis on the gills was approximately 70 % in the groups before treatment and the dead fish. After treatment the prevalence was 16.7 %. The density of *Cand.* Syngnamydia salmonis was 8.5 ± 4.7 before treatment and 5.1 ± 3.2 after treatment. The density was of *Cand.* Syngnamydia salmonis 8.5 ± 6.4 in the dead fish (Figure 3.35).
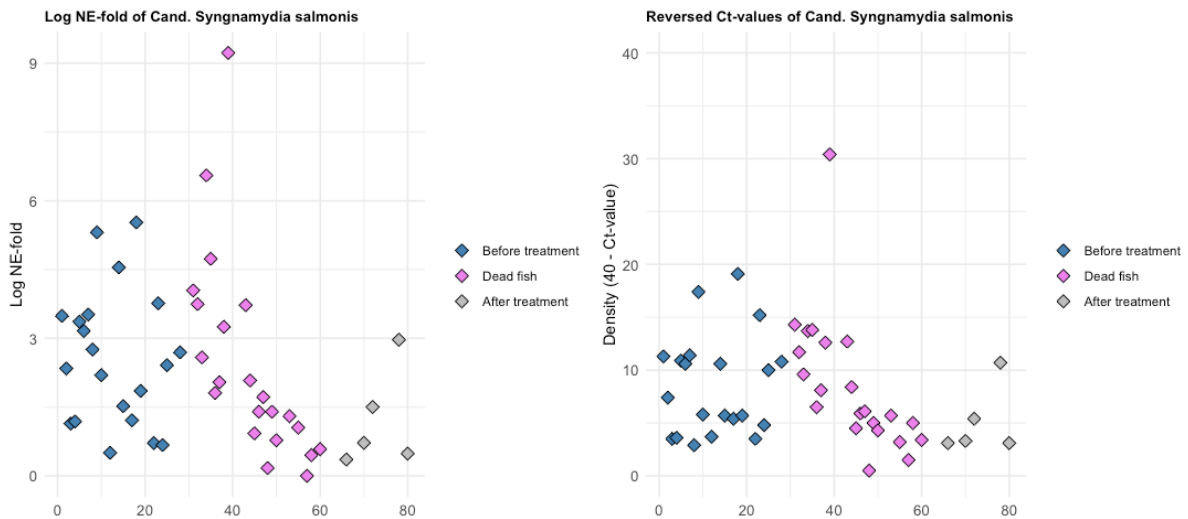
Figure 3.35: *Density of Candidatus Syngnamydia salmonis on the gills of salmon from before (blue dots) and after (grey dots) treatment, and in the dead fish (pink dots) at Location LB. The data are represented as Log NE-fold and reversed Ct-values (40 – Ct-value). N = 21 positive individuals before treatment, N = 5 positive individuals after treatment, and N = 22 positive individuals of the dead fish.*

The prevalence of *Candidatus* Branchiomonas cysticola was 100 % for all three sampling groups. The density of *Cand.* Branchiomonas cysticola was 25.2 ± 3 before treatment and 25.0 ± 2.5 after treatment. The density of *Cand.* Branchiomonas cysticola was 27.0 ± 2.4 in the dead fish (Figure 3.36).
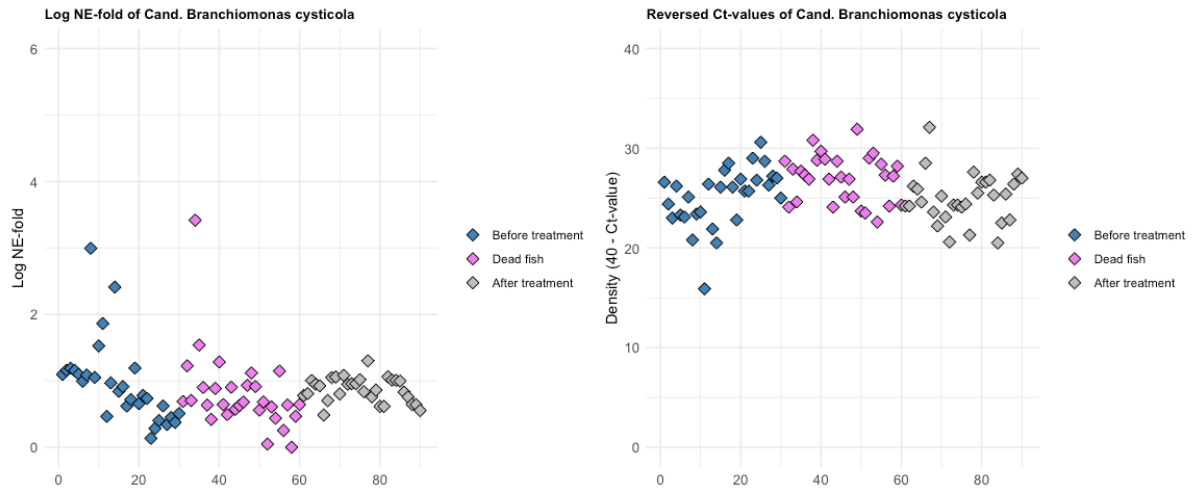
Figure 3.36: Density of Candidatus Branchiomonas cysticola on the gills of salmon from before (blue dots) and after (grey dots) treatment, and in the dead fish (pink dots) at Location LB. The data are represented as Log NE-fold and reversed Ct-values (40 – Ct-value). N = 30 positive individuals before treatment, N = 30 positive individuals after treatment, and N = 30 positive individuals of the dead fish.

The prevalence of Piscine orthoreovirus (PRV1) on the gills of salmon in all three sampling groups was 100 % on the gills. The density of PRV1 on the gills before treatment was 19.4 ± 2.6, whereas the density of PRV1 after treatment was 18.8 ± 1.7. The density of PRV1 in the dead fish was 19.1 ± 1.7 (Figure 3.37).
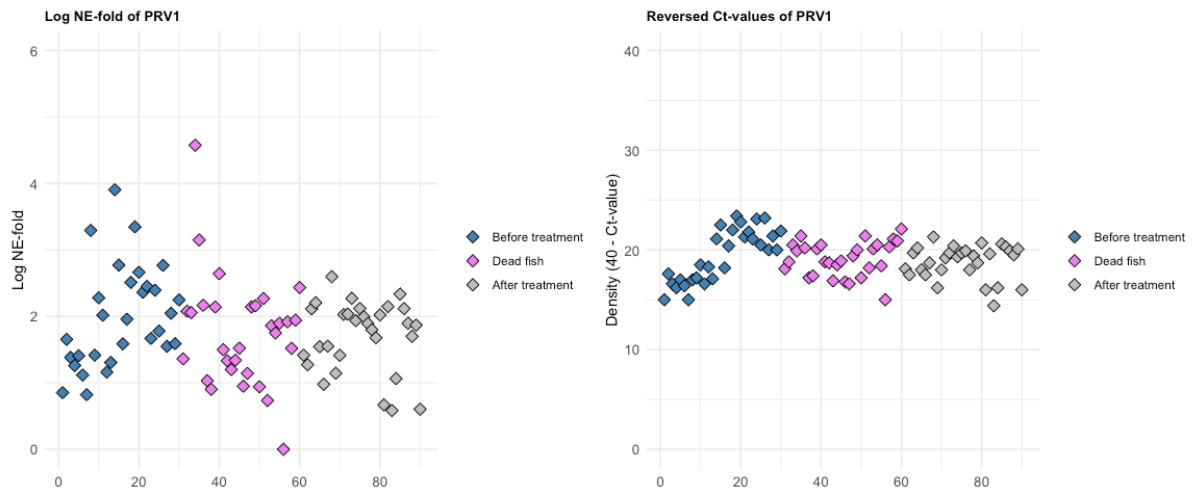
Figure 3.37: Density of Piscine orthoreovirus 1 (PRV1) on the gills of salmon from before (blue dots) and after (grey dots) treatment, and in the dead fish (pink dots) at Location LB. The data are represented as Log NE-fold and reversed Ct-values (40 – Ct-value). N = 30 positive individuals before treatment, N = 30 positive individuals after treatment, and N = 30 positive individuals of the dead fish.

The prevalence of Salmon gill poxvirus (SGPV) on the gills was 10 % before treatment and 16.7 % after treatment. The prevalence of SGPV was 70 % in the dead fish. The density of SGPV before treatment

was 7.0 ± 2.2, while the density after treatment was 4.9 ± 3.8. The density of SGPV was 9.6 ± 5.4 in the dead fish (Figure 3.38).

Figure 3.38: *Density of Salmon gill poxvirus (SGPV) on the gills of salmon from before (blue dots) and after (grey dots) treatment, and in the dead fish (pink dots) at Location LB. The data are represented as Log NE-fold and reversed Ct-values (40 – Ct-value). N = 3 positive individuals before treatment, N = 5 positive individuals after treatment, and N = 21 positive individuals of the dead fish.*

For all three sampling groups, the prevalence of *Paranucleospora theridion* on the gills was 100 %. The density of *P. theridion* before treatment was 19.1 ± 3.6, while the density after treatment was 19.5 ± 2.9. The density of *P. theridion* was 23.6 ± 4.0 in the dead fish (Figure 3.39).
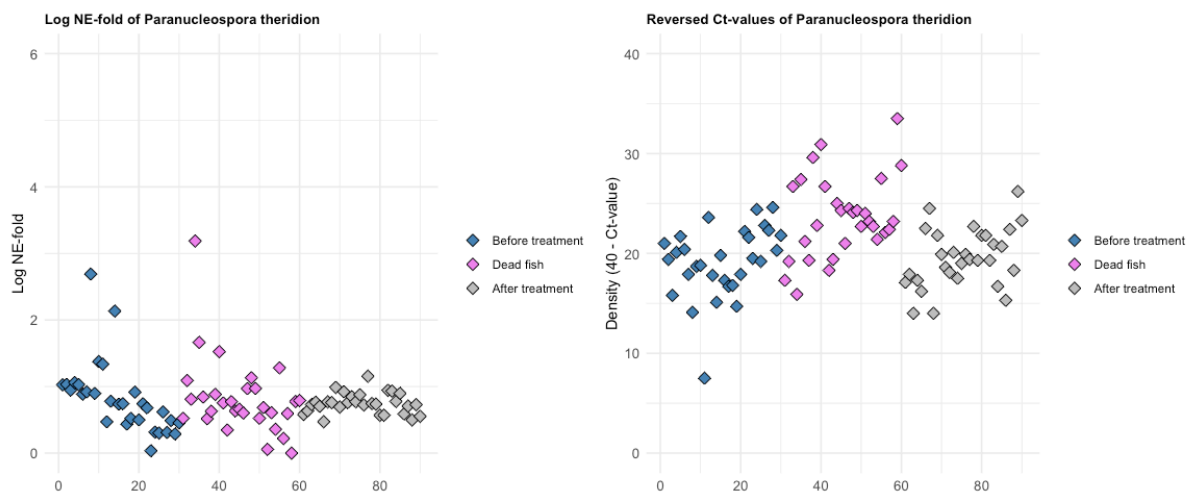
Figure 3.39: *Density of Paranucleospora theridion on the gills of salmon from before (blue dots) and after (grey dots) treatment, and in the dead fish (pink dots) at Location LB. The data are represented as Log NE-fold and reversed Ct-values (40 – Ct-value). N = 30 positive individuals before treatment, N = 30 positive individuals after treatment, and N = 30 positive individuals of the dead fish.*

As for *Tenacibaculum* spp. in the groups "before treatment" and "after treatment", the prevalence was 10 % and 16.7 %, respectively. The prevalence of *Tenacibaculum* spp. in the dead fish was 53.3 %. The density of *Tenacibaculum* spp. was low in all sampling groups (Figure 3.40).
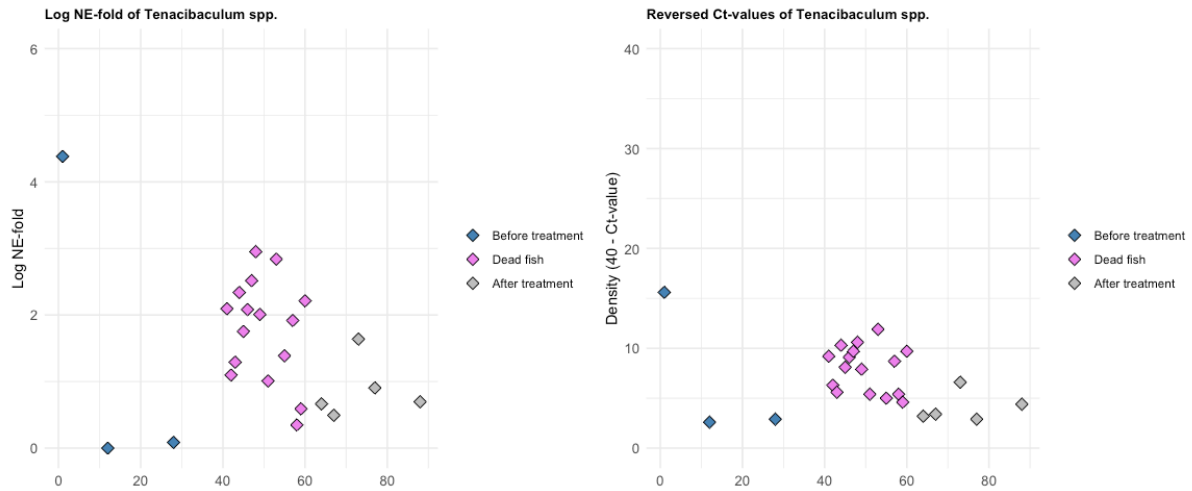
Figure 3.40: *Density of Tenacibaculum spp. on the gills of salmon from before (blue dots) and after (grey dots) treatment, and in the dead fish (pink dots) at Location LB. The data are represented as Log NE-fold and reversed Ct-values (40 – Ct-value). N = 3 positive individuals before treatment, N = 5 positive individuals after treatment, and N = 16 positive individuals of the dead fish.*

Most individuals were positive for Infectious pancreas necrosis virus (IPNV) in the kidney, and the prevalence (%) and density (40 – Ct-value) of IPNV for the kidney is presented below. The prevalence of IPNV in the kidney was 100 % before treatment and in the dead fish, and 93.3 % in the group after treatment. The average density of IPNV before treatment was 9.3 ± 2.3, while the average density after treatment was 9.0 ± 2.8. The average density of IPNV in the dead fish was 8.9 ± 2.6 (Figure 3.41).
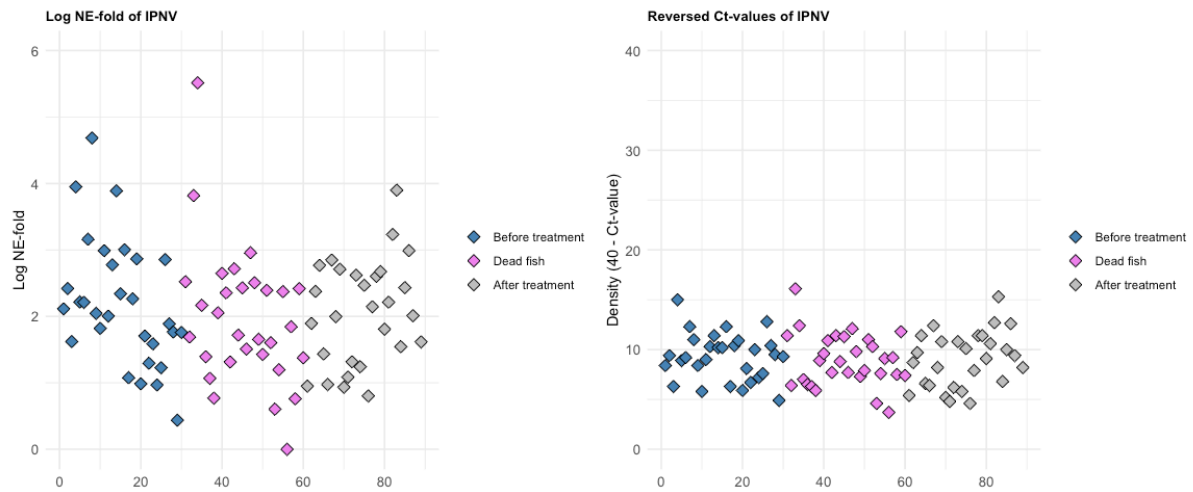
Figure 3.41: *Density of Infectious pancreatic necrosis virus (IPNV) in the kidney before (blue dots) and after (grey dots) treatment, and in the dead fish (pink dots) at Location LB. The data are represented as Log NE-fold and reversed Ct-values (40 – Ct-value). N = 30 positive individuals before treatment, N = 30 positive individuals after treatment, and N = 28 positive individuals of the dead fish.*

3.3.4 Histology

The mean histological gill score of the fish before and after treatment and of the dead fish is presented in Figure 3.42. Score 3 was given with the parameters for fresh aneurisms (parameter VII), bleeding aneurisms (parameter VIII) and necrosis (parameter XI) before treatment. Score 3 was given with bleeding aneurisms (parameter XIII) (Figure 3.45) after treatment (Figure 3.42). The most frequent parameters observed histologically both before and after treatment were hyperplasia of mucus cells (parameter I) (Figure 3.43) and hypertrophy of epithelial cells (parameter VIII) (Figure 3.44). Epitheliocystis was also observed on the secondary lamellae (Figure 3.44). Gill score 1 and 2 was mostly given, and few histological sections were given score 3 and 0.
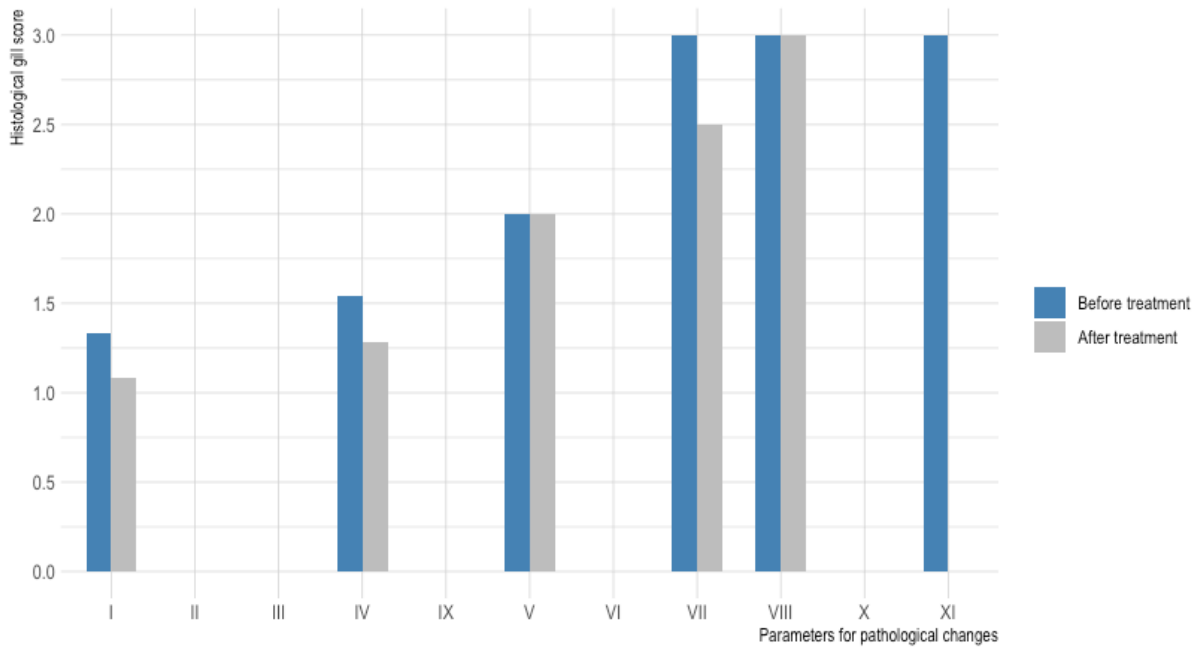
Figure 3.42: *Mean histological gill score of the fish before and after treatment at Location LB. Eleven pathological changes are included in this study on the gills of Atlantic salmon.* ***I:*** *Hyperplasia of mucus cells,* ***II:*** *Clubbing,* ***III:*** *Lifting,* ***IV:*** *Hypertrophy of epithelial cells,* ***V:*** *Hyperplasia of epithelial cells,* ***VI:*** *Thickened distal primary lamellae,* ***VII:*** *Fresh aneurisms,* ***VIII:*** *Bleeding aneurisms,* ***IX:*** *Old aneurisms,* ***X:*** *Inflammation,* ***XI:*** *Necrosis. N = 30 fish before treatment, N = 30 fish after treatment.*
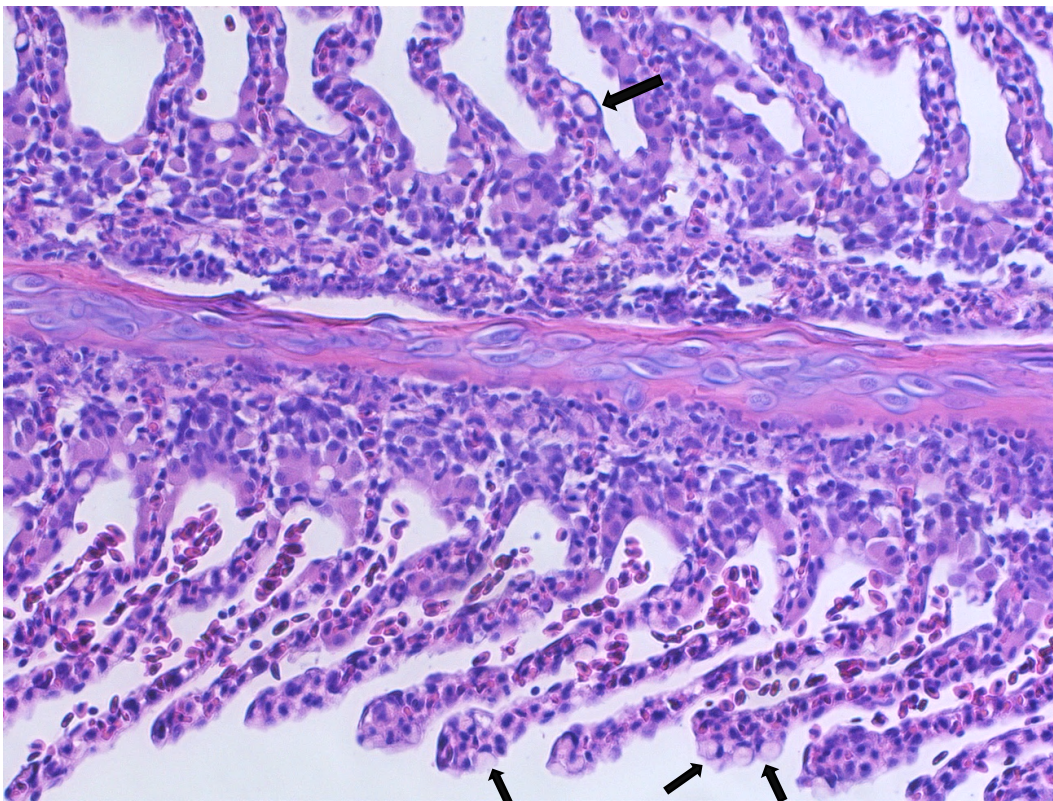
Figure 3.43: *Fish no. 20 (before treatment) with hyperplasia of mucus cells (black arrows) at Location LB.*
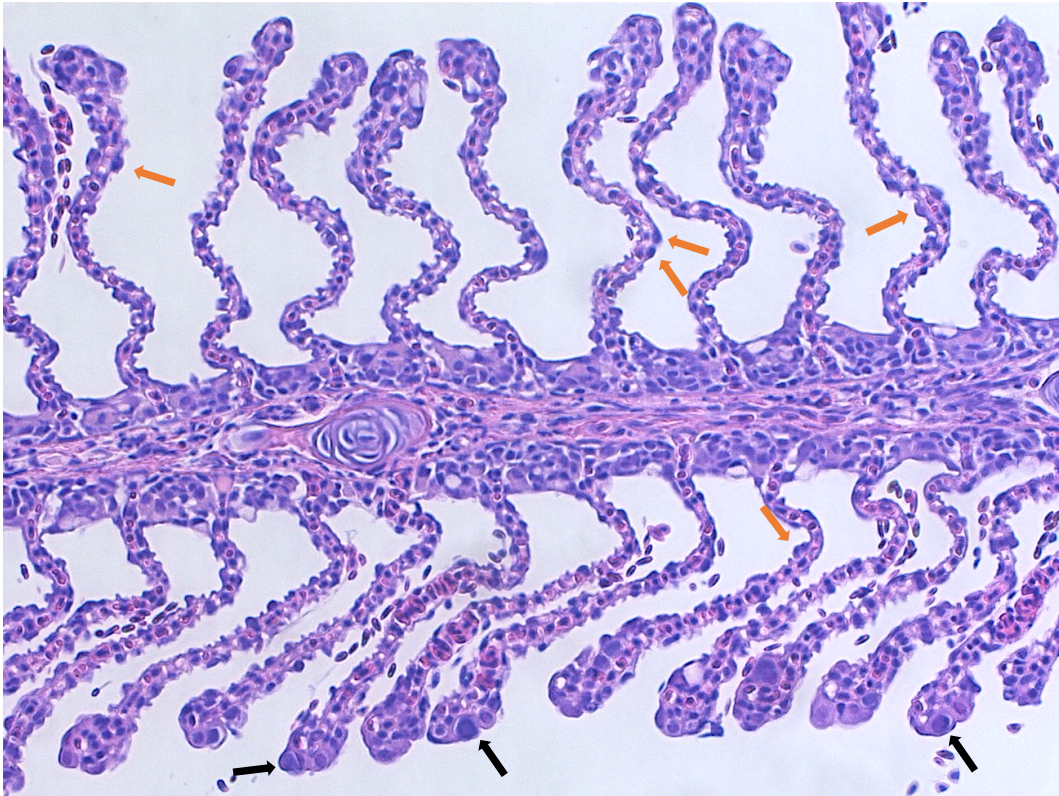
Figure 3.44: *Fish no. 17 (before treatment) with hypertrophic epithelial cells (orange arrows) and epitheliocysts (black arrows) at Location LB.*
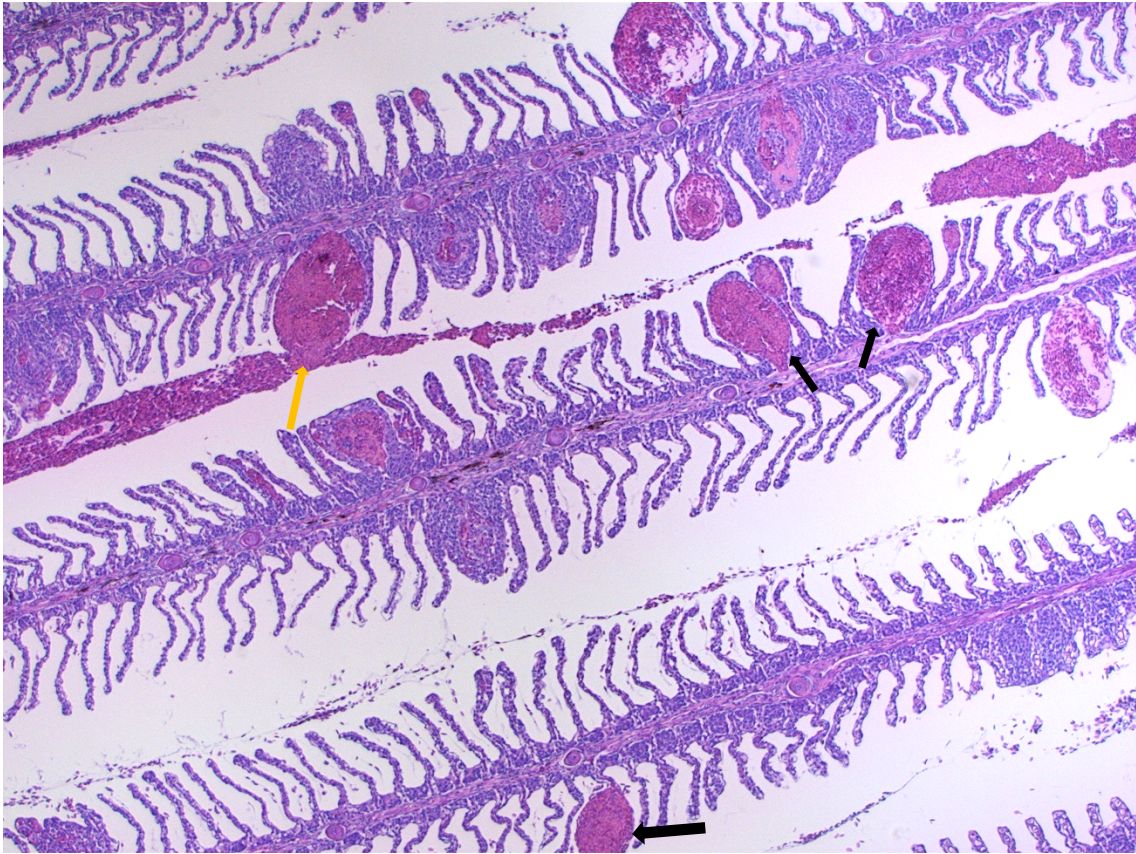
Figure 3.45: *Fish no. 61 (after treatment) with fresh aneurisms (black arrows) and bleeding aneurisms (orange arrow) at Location LB.*

4 Discussion

Amoebic gill disease (AGD) caused by *P. perurans* and sea lice infestations (*Lepeophtheirus salmonis* and *Caligus elongatus*) are two of the main challenges in Norwegian marine farmed Atlantic salmon (*Salmo salar*) (Abolofia and Wilen 2017; Barrett et al. 2022; O. M. V. Dahle et al. 2020; Johnsen et al. 1995; Powell and Kristensen 2014; Tröße et al. 2021). If farmed salmon is heavily infected, treatment against AGD and/or lice is necessary. In addition to the economic costs of the treatments, this challenges the fish health and welfare due to stress prior to treatment and the treatment itself (Ashley, Sneddon, and McCrohan 2007; Bui et al. 2022; Gismervik et al. 2019; Moltumyr et al. 2022; Nilsson et al. 2019; Nordgreen et al. 2009; Østevik et al. 2022; Mark D. Powell et al. 2015). It is therefore useful with increased knowledge about the impacts of treatment of farmed salmon to improve treatment routines and fish health- and welfare.

In this study, two treatment methods against AGD (*P. perurans*) and sea lice was conducted in commercial production of Atlantic salmon to outline the effects of treatment on the gills of salmon. To evaluate the effects, registration of macroscopic and histological gill score was included, registration of sea lice, visible pathology, mortality, and analysing gill- and kidney tissue by doing real-time RT-PCR. In addition, a challenge experiment was conducted to test the virulence of *P. perurans* cultured in different microbiota. Macroscopic gill score and real-time RT-PCR analysis were used to evaluate results from the challenge. Gill microbiome of marine salmon if not fully understood, but it has been shown differences in bacterial composition on the gills between AGD-negative and AGD-positive salmon by Bowman and Nowak (2004). The importance of a better understanding of the role of salmon gill microbiota is useful for future risk assessment of AGD (O. Benedicenti et al. 2019; Bovee et al. 1979; Bowman and Nowak 2004). When marine farmed salmon are treated with freshwater, a rapid change in the aqueous environment happens and it is not unlikely that this changes the gill microbiome. It is still unclear how this affects the gill health of farmed salmon.

Thermal delousing using Thermolicer is documented by Grøntvedt et al. (2015) to reduce the number of mobile and adult salmon lice. Optilicer has also shown to be an efficient delousing method (Grøntvedt et al. 2015; Roth 2016). Thermal treatment challenges however the fish welfare considering gill- and skin injuries, eye damages and brain haemorrhages that have been documented from laboratory trials and in the field (Bui et al. 2022; Gismervik et al. 2019; Nilsson et al. 2019; Østevik et al. 2022).

4.1 Challenge: The importance of gill microbiome for *P. perurans* infection on the gills of Atlantic salmon

In addition to temperature and salinity, variation in gill microbiome has shown to influence the virulence and density of *P. perurans* in AGD-infected salmon (O. Benedicenti et al. 2019; Bowden 2008; Bowman

and Nowak 2004; Clark and Nowak 1999; O. M. V. Dahle et al. 2020; Douglas-Helder et al. 2001; Douglas-Helders et al. 2003b; Hjeltnes, Karlsbakk, Tor Atle Mo, et al. 2014; Hvas, Karlsbakk, Maehle, et al. 2017; Johnsen and Jensen 1994; Ní Dhufaigh et al. 2021; Oldham et al. 2016; Slinger et al. 2021; Vollset et al. 2021). Knowledge about the bacterial composition on the gills of salmon is however still limited. In this study, difference in density and virulence of *P. perurans* when changing the bacterial composition in the culture media have been observed. This was also observed by Lyng (2021) when the H20/16Pp-clone was unable to establish a permanent infection on the gills of salmon in pure culture of *V. splendidus.*

In this study, the H02/13Pp-clone of *P. perurans* in pure culture of *V. splendidus* showed 100 % prevalence throughout the challenge period but was less virulent on the gills of salmon (lower gill score/less pathology) than the other groups challenged with H02/13Pp (HV-HVBM, HV-LVBM, HVBM-HV). *P. perurans* cultured in LVBM (bacteria isolated from culture medium with H20/16Pp clone) was able to establish infection on the gills of salmon and result in gill score, but the infection was not permanent. Loss of virulence in clones of *P. perurans* is likely due to loss of certain bacteria species in the culture media (O. M. V. Dahle et al. 2020; Tröße et al. 2021).

The fish in tank 2 (HV-HVBM) had the highest GS throughout the challenge. The prevalence of *P. perurans* was 100 % throughout the challenge in the groups challenged with H02/13Pp (HV-V.Spl., HV-HVBM, HV-LVBM, HVBM-HV), which correlates with the GS. Registration of GS at each sampling and doing real-time RT-PCR of the gill tissue made it possible to analyse the correlation between the GS and the density of *P. perurans*. GS was observed in *P. perurans*-negative salmon, indicating that the microbiota may be crucial for gill pathology. Such findings have also been observed by Downes et al. (2015) from marine farmed salmon in the field. GS indicates mucoid patches and detection of lesions on the gills but does not necessarily specify the aetiology of AGD. This usually requires examinations to detect the presence of *P. perurans* on the gills. One disadvantage of histological sections is that the amoebae can be washed away when fixating the sections. Hence, real-time RT-PCR can give low Ct-values of *P. perurans* even if there is no detection of the amoebae histologically (Adams, Ellard, and Nowak 2004; Downes et al. 2015; Nowak et al. 2002). Zilberg et al. (2001) recorded GS without detecting the amoeba histologically. Dahle et al. (2020) and Røed (2016) observed a positive correlation between the amount of amoeba and increased GS in their study.

Reduction of virulence of *P. perurans* (H02/13Pp) was observed in this study, likely due to loss of certain bacterial species in the culture medium. At 25 dpc, the GS was significantly higher in HV-HVBM compared to HV-V.Spl., but the amount of rRNA from the latter, based on qPCR, were higher (Figure 3.4 and Table 3.2). Loss of virulence of *P. perurans* has been seen in several studies, i.a. by Collins et al. (2017), Jellett and Scheibling (1988), Bridle et a. (2015), Bridle et al. (2012) and Cano et al. (2019).

Bridle et al. (2015) used clones of *P. perurans* that was virulent for 70 days after being cultured but lost its virulence after 3 years in clonal culture. It has been suggested that loss of virulence may be associated with the lack of extracellular products (Bridle et al. 2015; Cano et al. 2019; O. M. V. Dahle et al. 2020). Cano et al. (2019) passaged *P. perurans* 98 times *in vitro* before it lost its virulence, while Bridle et al. (2015) passaged *P. perurans* 200 times. Loss of virulence in other pathogens than *P. perurans* is not unusual (O. M. V. Dahle et al. 2020; Dorson, Castric, and Torchy 1978; Songe et al. 2014).

4.2 Freshwater treatment against AGD

The use of freshwater treatment against AGD was first described in the 1980's in Tasmania by Munday et al. (1990) and showed good effects against AGD (Birlanga et al., 2022; Munday et al., 2001; Oldham et al., 2016; Taylor et al., 2009; Wood et al., 2021). Location LA was diagnosed with gill disease with the detection of *P. perurans* and conducted therefore freshwater treatment against AGD autumn 2021. The treatment lasted for 4 hours at 14 °C.

Clark et al. (2002) used freshwater treatment for 2 hours at 14.9-16.4 °C and almost 90 % of the amoebae present on the gills were successfully removed. Reinfection of *P. perurans* occurred however within one week. Reinfection of *P. perurans* after freshwater baths have been recorded in several studies post freshwater treatment (Kube, Taylor, and Elliott 2012; Mccormack et al. 2021; Thoen et al. 2020). The sampling after treatment in this study was conducted 11 days after freshwater treatment. This gives *P. perurans* time to reoccur on the gills, or the treatment was not very efficient, as the prevalence of *P. perurans* was approximately 100 % in all sampling groups. In addition, Location LA had 11 sea cages, so infection between the cages after treatment was highly possibly, as all the sea cages were not treated at the same time. Parsons et al. (2001) treated for 3 hours at 20-22.6 °C. The number of amoebae decreased after the freshwater treatment and the prevalence of *P. perurans* was significant lower after treatment compared to before treatment. By looking at the histological sections, Parson et al. (2001) observed a higher number of amoebae in caverns after treatment than before and suggested that alternative methods and/or improvement related to the freshwater treatment was necessary for future risk assessment of AGD. A study by Hudson et al. (2022) compared freshwater treatment at 3 and 15 °C for 2 hours. *In vitro*, treatment at 3 °C was significantly more efficient than treatment at 15 °C and *P. perurans* lost attachment to their substrate, but there was no difference between the two temperatures *in vivo*. *P. perurans* was observed histologically in caverns in this study, which can be registered by the real-time RT-PCR.

Mortality of farmed salmon in relation to freshwater treatment has been as high as 50 % (Parsons et al. 2001). Overton et al. (2019) studied the salmon mortality between 2012 and 2017 and found that thermal delousing treatments caused the highest mortality of the treatment methods (thermal, mechanical,

hydrogen peroxide, medical) the month after treatment compared to the month before. Mortality after freshwater treatment is not fully known (Sviland Walde et al. 2021). In this study, it was not registered a significant increase in average mortality of the fish after the freshwater treatment. The mortality of the fish usually increases after treatment due to the stress when pumping and handling the fish prior to treatment, in addition to the treatment itself (Hvas, Nilsen, and Oppedal 2018; Krogh 1937; Marshall 2002). Lyng (2021) conducted freshwater treatment and recorded higher mortality (1500 fish) the day of treatment compared to this study with only 63 fish dead the day of treatment.

The freshwater treatment had a significant reducing effect on the individual average gill score before treatment compared to after treatment (p < 0.0001). In this study, the highest average individual gill score before treatment was 3.4, while after treatment the highest average individual gill score was 2.6. Parsons et al. (2011) observed clear differences between clear, medium and heavy gill scores before treatment compared to after freshwater treatment.

Real-time RT-PCR analysis of the gill samples showed the presence of several pathogens that may have affected the gill health of the salmon, such as *P. perurans*, *Ichthyobodo* spp., SGPV, *P. theridion*, *Tenacibaculum* spp., *Cand.* Branchimonas cysticola, *Cand.* Piscichlamydia salmonis and *Cand.* Syngnamydia salmonis. Many of the pathogens identified at Location LA were marine microparasites, and the freshwater treatment was expected to have good effect and reduce the amount of these pathogens.

All individuals sampled at Location LA were positive for PRV1 in the gill- and kidney tissue. The density (40 – Ct-value) on the gills was however 20.9 ± 2, indicating that some fish may suffer from HSMI, but this requires histopathological examinations of the heart and skeletal muscles to confirm or reject. High prevalence of *P. theridion* on the gills in all sampling groups was not unexpected, as the parasite is a typical autumn-disease at water temperatures between 13 – 17 °C (Sveen et al. 2012). Here, the treatment did not affect the density of this parasite. The prevalence of SGPV was higher in the dead fish compared to before treatment, indicating that this virus may have affected the mortality of the fish.

A lot of aneurisms were observed histologically before treatment, and the amount of aneurisms increased after treatment.

4.3 Use of cold freshwater before thermal delousing

This is the first study to document the effects of thermal delousing and of salmon kept in cold freshwater. Location LB kept the fish in cooled freshwater at 8 °C for 4 hours followed by thermal delousing at 30 °C (Δ = 22 °C) for 30 s. Previous studies exposing salmon to warm water have resulted in severe injuries in the skin, gills, eyes, fins, snout and/or brain (Bui et al. 2022; Gismervik et al. 2019; Moltumyr et al.

2022; Østevik et al. 2022). Østevik et al. (2022) treated salmon in the field for 28 s at 33.9 °C (Δ = 18.4 °C). Moltumyr et al. (2021), (2022) and Gismervik et al. (2019) studied salmon exposed to water temperatures at 34 °C. Gismervik et al. (2019) also investigated water temperatures at 36-38 °C, which led to acute injuries in the gills, eyes, brain, nasal cavity, and thymus in the salmon.

By analysing data from the Norwegian salmon aquaculture between 2012 and 2017, Overton et al. (2019) saw an increased mortality rate of the fish the month after sea lice treatment compared to the month before treatment. This was also estimated by Walde et al. (2021). On the day of treatment, 8 of 86 964 fish in the treated cage died. This is 0.009 % of fish stock in the treated cage. Kvale (2020) registered 0.4 % and 0.9 % mortality after thermal treatment, and many of the fish were observed with coagulated blood in the pericardial cavity. In this present study, only one fish of the dead fish was registered with petechial bleedings and one fish after treatment had skin lesions. The fish from this field work had few or less injuries than what has been observed in previous studies using mechanical treatments (Bui et al. 2022; Gismervik et al. 2019; Overton et al. 2019; Sviland Walde et al. 2021). The month before treatment, the mortality was 0.5 %, while the month after treatment the mortality was 0.6 %. At one of the locations in the study by Kvale (2020), the mortality was 2.2 % the month before treatment, and increased to 3.7 % the month after treatment. In addition, more severe external and internal pathology were observed by Kvale (2020) than in this study. The treatment in this study did not result in a significant increase in the average mortality of the fish, which is often observed at fish farms after treatment (Oliveira et al. 2021). The mortality registered in this study was lower than recorded by Bui et al. (2022) who treated fish two times at 27, 30 and 33 °C and recorded 5.3 % mortality when treating at 27 °C, 12.4 % mortality at 30 °C and 18.9 % mortality at 33 °C. Publications on mortality after treatment from the field is poorly documented.

Reduced appetite is expected after treatment, especially after acute elevated temperatures (Elliott, 1991; Moltumyr et al., 2022), but according to Poppe et al. (2018), the documentation of the effects of thermal treatment on fish health is not sufficient. The fish welfare in the treated sea cage was considered good before, during and after treatment, as the fish started eating the same day of treatment again. It often takes a few days after treatment until the fish starts feeding again, but this is not well documented.

The prevalence of PRV1, *P. theridion* and *Cand.* Branchiomonas cysticola was still high after treatment, possibly explaining no significant difference in GS between the groups «before treatment» and "after treatment" in this study. The prevalence of *Cand.* Syngnamydia salmonis, *P. theridion* and *Ichthyobodo* spp. was high in the fish before treatment and in the dead fish and may have contributed to the mortality of these fish groups. The prevalence of *Cand.* Syngnamydia salmonis and *P. perurans* was reduced after treatment; *Cand.* Syngnamydia salmonis can multiply in both gill epithelial cells and in *P. perurans* (Nylund, Pistone, et al. 2018). Reduced density after treatment can therefore be a result of reduced

density of *P. perurans*. This most likely indicates that the main amount of this bacteria is present in *P. perurans* and not on the gills of salmon. Gunnarsson et al. (2017) recorded high levels of PRV with GDs, which corresponds with the findings of PRV1 at both locations in this study. The prevalence of IPNV in the kidney was nearly 100 % in all fish groups, but the mean Ct-value was high (30.9 ± 2.6). This implies carrier status for the fish, and the fish might have had an IPN-outbreak during the freshwater phase. The prevalence of ISAV, SGPV and *Ichthyobodo* spp. was higher in the dead fish than in the groups before and after treatment, which may have affected the mortality of the dead fish.

5 Conclusion and future perspective

By changing the composition of microbiota in the culture media or on the gills of salmon, it is possible to change the virulence of *P. perurans* clones. The reason for why individual clones loses their virulence when culturing *P. perurans* over time may be related to the composition of bacteria in the culture media. What remains now is to identify what bacteria/bacterial species are the contributing cause to why clones of *P. perurans* result in AGD with subsequent mortality. A better understanding of gill pathogens and their association with environmental factors is useful for future risk assessment and management of AGD. The importance of good gill health is crucial in the production of Atlantic salmon, as the gills are an organ with multiple crucial functions.

The field studies showed that other pathogens than *P. perurans* (*P. theridion,* PRV1, *Cand.* Branchiomonas cysticola, *Cand.* Piscichlamydia salmonis, *Cand.* Syngnamydia salmonis, SGPV, IPNV, ISAV HRP0) may contribute to the mortality of farmed salmon. To what extent the pathogens influenced the mortality of the fish during treatment is not entirely certain. Treating fish against AGD does not necessarily result in a significant reduced gill score due to other gill pathogens, but the treatments in this study had a significant reducing effect on the number of sea lice. The handling and pumping prior to treatment are stressful for the fish and can contribute to increased mortality.

The health status of the fish prior to treatment is important for fish health personnel to evaluate to avoid as much mortality as possible. This is also important for a more sustainable production of farmed salmon with the regards of fish health- and welfare. Only histological sections of the gills were analysed in this study, but other organs such as the brain, skin, eyes, would also be interesting to investigate, as Gimservik et al. (2019) did in their study. It would also be interesting to follow the treated salmon in this field study for a longer period to analyse the pathogen load and long-term effects of the treatments.

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7 Appendix

Table 7.1: *Average and range of Ct-values and prevalence (%) of the analysed assays for from the field, Location LA. G = gill, K = kidney.*

	Before treatment			Dead fish			After treatment		
	Ct-value		Prevalence (%)	Ct-value		Prevalence (%)	Ct-value		Prevalence (%)
	Average	Range		Average	Range		Average	Range	
ELA1A (G)	14.1	1.0 – 15.8	-	14.9	13.6 – 17.0	-	15.8	14.2 – 16.9	-
ELA1A (K)	14.8	13.3 – 16.1	-	17.5	15.8 – 18.9	-	15.5	13.4 – 19.7	-
Salmonid Alphavirus (G)	Neg	Neg	0	Neg	Neg	0	Neg	Neg	0
Salmonid Alphavirus (K)	Neg	Neg	0	Neg	Neg	0	Neg	Neg	0
Infectious salmon anemia virus (G)	36.3	35.7 – 36.9	13.3	Neg	Neg	0	Neg	Neg	0
Infectious salmon anemia virus (K)	Neg	Neg	0	Neg	Neg	0	Neg	Neg	0
Piscine orthoreovirus 1 (G)	23.1	18.7 – 25.9	100	26.5	23.4 – 30.0	100	21.8	18.8 – 26.4	100
Piscine orthoreovirus 1 (K)	21.0	16.7 – 24.1	100	25.5	23.5 – 27.9	100	23.7	21.2 – 26.2	100
Piscine myocarditis virus (G)	Neg	Neg	0	Neg	Neg	0	Neg	Neg	0
Piscine myocarditis virus (K)	Neg	Neg	0	Neg	Neg	0	Neg	Neg	0
Salmon gill poxvirus (G)	29.8	19.9 – 35.9	46.7	29.2	18.4 – 36.9	10	26.3	23.1 – 33.9	100
Infectious pancreas necrosis virus (G)	Neg	Neg	0	Neg	Neg	0	Neg	Neg	0
Infectious pancreas necrosis virus (K)	Neg	Neg	0	36.1	35.8 – 36.5	10	36.7	36.4 – 37.2	16.7
Paranucleospora theridion (G)	15.1	10.8 – 18.0	100	12.2	2.6 – 17.7	100	17.6	13.4 – 22.9	100
Paranucleospora theridion (K)	16.9	13.1 – 20.7	100	13.8	7.6 – 21.5	100	18.0	13.5 – 21.8	100
Paramoeba perurans (G)	14.3	10.5 – 19.3	100	21.4	16.1 – 35.3	95	24.3	15.8 – 30.5	96.7
Ichthyobodo spp. (G)	25.5	22.3 – 28.9	100	21.4	19.8 – 22.9	10	26.6	24.0 – 28.8	20
Perkinsela-like symbiont (G)	Neg	Neg	0	Neg	Neg	0	Neg	Neg	0
Tetracapsuloides bryosalmonae (G)	Neg	Neg	0	Neg	Neg	0	Neg	Neg	0
Tetracapsuloides bryosalmonae (K)	Neg	Neg	0	Neg	Neg	0	Neg	Neg	0
Parvicapsula pseudobranchicola (G)	37.7	37.7 – 37.8	6.7	Neg	Neg	0	Neg	Neg	0
Candidatus Branchiomonas cysticola (G)	19.9	16.6 – 24.4	100	18.1	14.0 – 20.9	100	18.1	14.4 – 32.3	100
Candidatus Syngnamydia salmonis (G)	22.4	18.7 – 27.3	100	27.0	17.8 – 32.7	100	31.6	22.5 – 37.6	90
Candidatus Piscichlamydia salmonis (G)	23.6	18.3 – 32.2	100	19.5	15.7 – 28.4	100	23.4	18.3 – 32.3	100
Tenacibaculum spp. (G)	34.5	28.6 – 37.3	63.3	34.4	24.5 – 37.8	75	35.4	32.9 – 37.0	30

Table 7.2: *Average and range of Ct-values and prevalence (%) of the analysed assays for from the field, Location LB. G = gill, K = kidney.*

	Before treatment		Prevalence (%)	Dead fish		Prevalence (%)	After treatment		Prevalence (%)
	Ct-value			Ct-value			Ct-value		
	Average	Range		Average	Range		Average	Range	
ELA1A (G)	16.4	13.4 – 27.6	-	15.6	12.9 – 24.4	-	15.8	14.6 – 17.2	-
ELA1A (K)	14.3	13.5 – 15.2	-	16.3	14.8 – 22.6	-	14.5	13.7 – 15.4	-
Salmonid Alphavirus (G)	Neg	Neg	0	Neg	Neg	0	Neg	Neg	0
Salmonid Alphavirus (K)	Neg	Neg	0	Neg	Neg	0	Neg	Neg	0
Infectious salmon anemia virus (G)	34.3	27.7 – 37.0	26.7	33.1	27.5 – 36.7	93.3	32.6	27.3 – 36.5	10
Infectious salmon anemia virus (K)	Neg	Neg	0	34.4	30.1 – 36.5	40	Neg	Neg	0
Piscine orthoreovirus 1 (G)	20.6	16.6 – 25.0	100	20.9	17.9 – 25.0	100	21.2	18.7 – 25.6	100
Piscine orthoreovirus 1 (K)	19.6	16.6 – 22.5	100	20	16.2 – 23.1	100	22.4	18.8 – 24.8	100
Piscine myocarditis virus (G)	Neg	Neg	0	36.6	36.4 – 36.8	6.7	Neg	Neg	0
Piscine myocarditis virus (K)	Neg	Neg	0	Neg	Neg	0	Neg	Neg	0
Salmon gill poxvirus (G)	33.0	31.0 – 35.3	10	30.4	17.4 – 37.3	70	35.1	28.4 – 36.9	16.7
Infectious pancreas necrosis virus (G)	34.7	31.9 – 39.3	26.7	34.5	31.9 – 36.6	33.3	34.5	34.7 – 35.6	10
Infectious pancreas necrosis virus (K)	30.7	25.0 – 35.1	100	31.1	23.9 – 36.3	100	31.0	24.7 – 35.4	93.3
Paranucleospora theridion (G)	20.9	15.4 – 32.5	100	16.4	6.5 – 24.1	100	20.5	13.8 – 26.0	100
Paranucleospora theridion (K)	19.5	15.5 – 24.5	100	17.6	6.3 – 25.5	100	19.6	13.2 – 29.9	100
Paramoeba perurans (G)	27.2	15.5 – 34.3	100	28.9	20.3 – 33.9	56.7	31.9	22.7 – 37.9	53.3
Ichthyobodo spp. (G)	30.4	22.9 – 35.0	36.7	29.8	24.0 – 35.5	80	31.7	18.9 – 36.6	56.7
Perkinsela-like symbiont (G)	Neg	Neg	0	Neg	Neg	0	Neg	Neg	0
Tetracapsuloides bryosalmonae (G)	Neg	Neg	0	Neg	Neg	0	Neg	Neg	0
Tetracapsuloides bryosalmonae (K)	Neg	Neg	0	Neg	Neg	0	Neg	Neg	0
Parvicapsula pseudobranchicola (G)	Neg	Neg	0	Neg	Neg	0	Neg	Neg	0
Candidatus Branchiomonas cysticola (G)	14.8	9.4 – 24.1	100	13.0	8.1 – 17.4	100	15.0	7.9 – 19.5	100
Candidatus Syngnamydia salmonis (G)	31.5	20.9 – 37.1	70	31.5	9.6 – 39.5	73.3	34.9	29.3 – 36.9	16.7
Candidatus Piscichlamydia salmonis (G)	Neg	Neg	0	35.0	32.6 – 37.4	6.7	Neg	Neg	0
Tenacibaculum spp. (G)	33.0	24.4 – 37.4	10	32.0	28.1 – 35.4	53.3	35.6	33.4 – 37.1	16.7
Yersinia ruckeri (G)	Neg	Neg	0	Neg	Neg	0	Neg	Neg	0

Table 7.3: *Average and range of Ct-values and prevalence (%) of P. perurans assay analysed for in the tissue samples in the challenge experiment.*

	11 dpc			18 dpc			25 dpc		
	Ct-value		Prevalence	Ct-value		Prevalence	Ct-value		Prevalence
	Average	Range	%	Average	Range	%	Average	Range	%
HV-V.spl.	22.6	16.1 - 29.9	100	23.2	19.6 - 26.2	100	22.9	12.6 - 27.4	100
HV-HVBM	17.2	14.4 - 20.8	100	20.8	16.7 - 25.1	100	21.4	16.1 - 26.1	100
LV-LVBM	Neg	Neg	0	Neg	Neg	0	Neg	Neg	20
LV-HVBM	32.9	27.6 - 36.5	80	Neg	Neg	0	Neg	Neg	0
HVBM	Neg	Neg	0	35.8	33.6 - 37.5	46.7	Neg	Neg	0
HV-LVBM	21.8	16.8 - 25.8	100	22.7	18.4 – 27.0	100	22	16.5 - 26.7	100
HVBM-LV	Neg	Neg	20	35.6	33.7 - 38.6	86.7	Neg	Neg	0
HVBM-HV	23.6	15.9 - 28.7	100	26.5	20.5 - 34.7	100	24.8	19.8 - 27.9	100
MYA	34.98	33.3 - 36.4	50	Neg	Neg	0	Neg	Neg	0

Table 7.4: *Average Ct-values and prevalence (%) of the Perkinsela-like symbiont assay analysed for in the tissue samples in the challenge experiment.*

	11 dpc			18 dpc			25 dpc		
	Ct-value		Prevalence	Ct-value		Prevalence	Ct-value		Prevalence
	Average	Range	%	Average	Range	%	Average	Range	%
HV-V.spl.	28.8	22.4 - 31.5	90	30.6	26.7 - 34.5	100	30.3	20.5 – 34.0	100
HV-HVBM	23.6	20.6 – 28.0	100	27.6	23.5 - 32.1	100	27.4	20.9 - 31.6	100
LV-LVBM	Neg	Neg	0	39.6	Neg	6.7	Neg	Neg	0
LV-HVBM	36.4	35.2 - 37.4	30	38.9	Neg	6.7	Neg	Neg	0
HVBM	Neg	Neg	0	Neg	Neg	0	34.4	32.0 - 36.8	13.3
HV-LVBM	28.6	22.7 - 32.5	100	29	24.0 - 34.8	100	29.2	23.6 - 35.3	100
HVBM-LV	38.7	38.2 - 39.2	20	37.2	36.9 - 37.5	10	37.9	37.5 - 38.3	13.3
HVBM-HV	32	23.5 - 39.2	100	31.8	26.9 - 36.8	93.3	31	25.9 - 34.4	100
MYA	37	36.1 - 37.8	50	Neg	Neg	0	Neg	Neg	0

Table 7.5: *Average Ct-values and prevalence (%) of the Candidatus Syngnamydia salmonis assay analysed for in the tissue samples in the challenge experiment.*

	11 dpc			18 dpc			25 dpc		
	Ct-value		Prevalence	Ct-value		Prevalence	Ct-value		Prevalence
	Average	Range	%	Average	Range	%	Average	Range	%
HV-V.spl.	Neg	Neg	0	34.6	34.6 -	6.7	36.3	36.3 -	6.7
HV-HVBM	35.5	35.5 -	10	34.5	34.0 - 35.1	13.3	34.8	34.8 -	13.3
LV-LVBM	35.1	33.7 - 36.5	20	Neg	Neg	0	Neg	Neg	0
LV-HVBM	36.7	36.7 -	10	Neg	Neg	0	Neg	Neg	0
HVBM	Neg	Neg	0	37.0	37.0 -	6.7	Neg	Neg	0
HV-LVBM	Neg	Neg	0	37.0	37.0 -	6.7	35.9	35.9 -	7.7
HVBM-LV	Neg	Neg	0	36.2	36.2 -	6.7	Neg	Neg	0
HVBM-HV	37.8	37.8 -	10	Neg	Neg	0	34.9	33.5 - 36.3	13.3
MYA	Neg	Neg	0	31.3	31.2 -	6.7	Neg	Neg	0

Table 7.6: *P-values for the density (40 – Ct-value) of the positive assays from Location LA after the nonparametric Kruskal-Wallis test.*

Pathogen	Before treatment vs. dead fish	Before treatment vs. after treatment	Dead fish vs. after treatment
Piscine orthoreovirus 1 (gills)	0.3739	0.3769	0.6241
Piscine orthoreovirus 1 (kidney)	0.5256	0.2988	0.5718
Salmon gill poxvirus	0.6045	0.4478	0.5966
P. theridion (gills)	0.4783	0.2022	0.4502
P. theridion (kidney)	0.5047	0.4516	0.3337
P. perurans	0.4113	0.3798	0.3898
Ichthyobodo spp.	< 0.0001	< 0.0001	0.2854
Cand. Branchiomonas cysticola	0.3594	0.3115	0.4177
Cand. Syngnamydia salmonis	0.3646	0.3564	0.5157
Cand. Piscichlamydia salmonis	0.3804	0.3753	0.4912
Tenacibaculum spp.	0.7951	0.007	0.005

Table 7.7: *P-values for the density (40 – Ct-value) of the positive assays from Location LB after the nonparametric Kruskal-Wallis test.*

Pathogen	Before treatment vs. dead fish	Before treatment vs. after treatment	Dead fish vs. after treatment
ISAV HPR0 (gills)	< 0.0001	0.7684	< 0.0001
Piscine orthoreovirus 1 (gills)	0.4228	0.4268	0.3567
Piscine orthoreovirus 1 (kidney)	0.5231	0.4795	0.3056
Salmon gill poxvirus	< 0.0001	0.8875	< 0.0001
Infectious pancreas disease virus (kidney)	0.4544	0.5024	0.5896
P. theridion (gills)	0.4311	0.4244	0.3002
P. theridion (kidney)	0.4651	0.4727	0.5011
P. perurans	< 0.0001	< 0.0001	0.4853
Ichthyobodo spp.	< 0.005	0.3992	0.4093
Cand. Branchiomonas cysticola	0.5409	0.3653	0.53
Cand. Syngnamydia salmonis	0.2892	< 0.0001	0.0001
Tenacibaculum spp.	0.0005	0.986	0.0001