Gill infections, gill pathology and gill-related mortality in farmed Atlantic salmon

Gjelleinfeksjoner, gjellepatologi og gjellerelatert dødlighet hos oppdrettslaks

Liv Østevik

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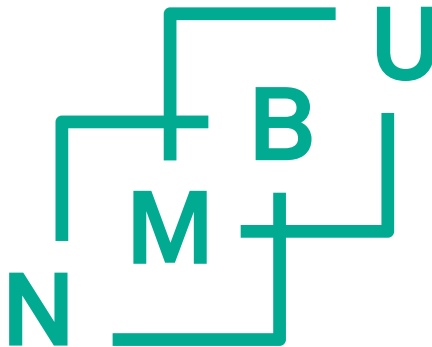
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Philosophiae Doctor (PhD) Thesis

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Seeing is believing

Acknowledgements

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1   Abbreviations and definitions

AGD – Amoebic gill disease

CGD – Complex gill disease

Fish-group - Fish from the same hatchery transferred to sea at the same time and to the same cage

FT - Flow through system - A system where the water supplied is used once and then discharged with or without treatment

HABs – Harmful algal blooms

IHC - Immunohistochemistry

ISH – *In situ* hybridization

mRNA - Messenger ribonucleic acid

Pathogen load – A term used to describe the amount of microbial genetic material detected in or on the gills of fish with PCR-analysis.

PGD - Proliferative gill disease

PGI – Proliferative gill inflammation

RAS – Recirculating aquaculture system - A closed or partially closed system in which the effluent water from the system is treated to enable reuse.

RT-qPCR - Reverse transcription quantitative polymerase chain reaction

Site - A cluster of pens that are managed from the same feed station

S1 - Smolt transferred to sea the year following start of feeding; S1 smolt are generally sea transferred in the spring

S0 - Smolt transferred to sea the same year as start of feeding; S0 smolt are sea transferred in the fall

SGPV – Salmon gill poxvirus

SGPVD – Salmon gill poxvirus disease

2   List of papers

Paper I
Liv Østevik, Marit Stormoen, Hege Hellberg, Marianne Kraugerud, Farah Manji, Kai-Inge Lie, Ane Nødtvedt, Hamish Rodger, Marta Alarcón,
A cohort study of gill infections, gill pathology and gill-related mortality in sea farmed Atlantic salmon (*Salmo salar* L.): Descriptive analysis
Journal of Fish Diseases, 00, 1– 21.
https://doi.org/10.1111/jfd.13662

Paper II
Liv Østevik, Marit Stormoen, Ane Nødtvedt, Marta Alarcón, Kai-Inge Lie, Andreas Skagøy, Hamish Rodger,
Assessment of acute effects of *in situ* net cleaning on gill health of farmed Atlantic salmon (*Salmo salar* L),
Aquaculture, Volume 545, 2021, 737203, ISSN 0044-8486,
https://doi.org/10.1016/j.aquaculture.2021.737203

Paper III
Liv Østevik, Marit Stormoen, Øystein Evensen, Cheng Xu, Kai-Inge Lie, Ane Nødtvedt, Hamish Rodger, Andreas Skagøy, Farah Manji, Marta Alarcón,
Effects of thermal and mechanical delousing on gill health of farmed Atlantic salmon (*Salmo salar* L.),
Aquaculture, Volume 552, 2022, 738019, ISSN 0044-8486,
https://doi.org/10.1016/j.aquaculture.2022.738019

3   Sammendrag

Det døde 54 millioner laks ved norske matfiskanlegg i 2021. Dette utgjør 15,5% av all laks som ble overført til lokaliteter i sjø. Gjellesykdommer en av de viktigste årsakene til økt dødelighet, økonomisk tap og redusert fiskevelferd ved oppdrett i sjø. Hovedmålet for doktorgradsarbeidet var å undersøke betydningen av og samspillet mellom utvalgte infeksiøs agens, miljøpåvirkninger og produksjonsforhold på gjellehelsen til oppdrettslaks. For å undersøke om variasjon i prevalens og mengde av infeksiøse agens og zoo- og fytoplankton påvirket gjellehelsen ble det gjennomført en prospektiv kohortstudie. I tillegg ønsket vi å beskrive utviklingen av gjelleinfeksjoner over tid i sjø. Grupper av laks i produksjonsområde 4, 5 og 6 ble regelmessing prøvetatt i løpet av settefiskfasen og det første året i sjø, og det ble samlet inn relevant produksjonsdata. *Neoparamoeba perurans*, fremsto som viktigste årsaken til gjellepatologi i de undersøkte kohortene. Det var ingen konsistent kovariasjon og kun svak eller ingen assosiasjon mellom omfanget av gjellepatologi og prevalens og mengde av SGPV, *Ca.* B. cysticola og *D. lepeopthterii* påvist ved PCR-analyse. Eneste unntak var assosiasjonen mellom epitelnekrose/apoptose og SGPV, og i mindre grad *Ca.* B. cysticola. Variasjon i zoo- og fytoplankton-nivåer i vannet viste ingen sammenheng med gjellesykdom eller totaldødelighet i våre kohorter. Ko-infeksjoner med tre eller flere agens var vanlig ved alle sjøanlegg. For å undersøke om notvask og termisk og mekanisk avlusning kan ha akutte effekter på gjellehelsen hos oppdrettslaks ble det gjennomført tre ulike historiske kontroll feltstudier. Det ble funnet en økning i antall fisk med subakutte karskader, hovedsakelig tromber, første dag etter notvask av nøter med moderat begroing. Det var en økning i omfanget av karskader og epitelhyperplasi i gjelle etter både mekanisk og termisk avlusning, selv om omfanget av gjellevev med slike forandringer var relativt lavt. Det ble funnet en økning i antall fisk med ulike gjellepatogener observert histologisk etter begge behandlingsmetodene. Etter termisk avlusning fant vi en økning i mengde *Ca.* B. cysticola arvestoff påvist ved PCR og endret genuttrykk av gener som er involvert i cellestress, betennelse, reparasjon og proliferasjon.

4 English summary

Last year (2021) 54 million Atlantic salmon died at Norwegian food producing sea sites. This constitutes a loss of 15,5% of the fish that were transferred to sea. Gill diseases are among the health issues that causes considerable mortality, economic loss, and reduced fish welfare in salmonid mariculture. The overall aim of the Ph.D. work was to investigate the impact and interactions of selected microorganisms, environmental and managerial factors on gill health of farmed Atlantic salmon. To determine if variation in pathogen prevalence and load, and zoo- and phytoplankton levels had an impact on gill health we performed a prospective cohort study. Groups of Atlantic salmon in Western Norway were followed with repeated sampling and data collection from the hatchery phase and throughout the 1st year at sea. A secondary goal was to describe the temporal development of selected gill infections. *Neoparamoeba perurans* appeared to be the most important cause of gill pathology in the study cohorts. No consistent covariation and no or weak associations were observed between the extent of gill pathology and prevalence or load of SGPV, *Ca.* B. cysticola and *D. lepeophtherii.* The only exception was an association between epithelial necrosis/apoptosis and SGPV, and to a lesser extent *Ca.* B. cysticola. At sea, *D. lepeophtherii* and *Ca.* B. cysticola persistently infected all fish-groups, while *N. perurans* and SGPV infection showed a seasonal variation. There was no impact of zoo- and phytoplankton on gill health or overall mortality in our cohorts. Co-infection with three or more putative gill pathogens was common across all sites. To determine if *in situ* net cleaning, thermal and mechanical delousing had an impact on gill health, we performed three separate historical control field trials. Exposure to biofouling debris during a single *in situ* net cleaning event of moderately fouled net pens was associated with an increase in the number of salmon with thrombi in the gills. There was an increase in vascular and hyperplastic gill lesions observed post-treatment after a single thermal or mechanical delousing treatment, though the overall percentage of gill tissue with pathology was generally low. An increased pathogen load of *Ca.* B. cysticola and differential expression of genes involved in pathways of cell stress, inflammation, repair, and proliferation was detected in the gill tissue after the thermal delousing event. Furthermore, there was an increase in the number of fish with microorganisms and lesions possibly associated with pathogens observed in the gill tissue after both treatments.

5   Synopsis

5.1   Introduction

5.1.1   Salmonid aquaculture and gill disease

Aquaculture is an important industry in Norway. Farmed Atlantic salmon (*Salmo salar* L.) of a total value of 138 billion NOK were slaughtered and sold in 2020 (Fiskeridirektoratet, 2021). Salmonids and predominantly Atlantic salmon are the main species cultured in Norway. Norwegian salmon production sites generally belong to one of three categories: Broodfish, hatchery or sea sites. Broodfish sites supply and produce fertilized eggs. Hatchery sites (also referred to as freshwater sites in this thesis) are where eggs hatch and fry grow until smoltification and sea transfer. Lastly, fish are kept at sea sites from the smolt stage until they are ready for slaughter (Figure 1). The time from fertilised egg to slaughter takes about 24-36 months for farmed fish.
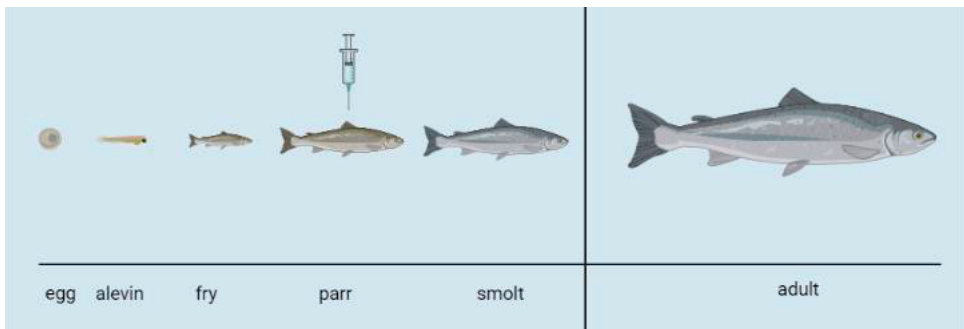
Figure 1. Life cycle of Atlantic salmon destined for slaughter. *Fertilized eggs are provided from brood stock facilities. Alevins (yolk-sac larva) survive on the yolk sac until they start feeding and become fry. Fry then become parr. Parr later undergo smoltification to become smolt at which time they are transferred to sea cages at food-producing sea sites. Vaccination is undertaken prior to sea transfer. (Figure created in BioRender.com).*

Norwegian salmonid aquaculture has undergone continuous development since its start in the 1960s and 70s. There has been a shift from smaller farms and net pens in fjords to larger farms and net pens located further out in the fjords or at sea. This has resulted in more exposure to currents and waves as well as higher levels of water exchange. In recent years land-based facilities where fish are kept from hatching or smoltification until slaughter have been built, but currently only a fraction of the Norwegian salmon produced originate from such facilities. Vaccines developed against several bacterial diseases have successfully reduced the impact of these diseases (Gudding & Goodrich, 2014). However, the intensive production and high density of fish is still associated with a range of infectious and non-infectious health problems. In 2021 a total of 54 million Atlantic salmon died at food producing sea sites (Sommerset et al., 2022). Infectious agents remain the most important causes of disease and the diseases caused by bacteria, viruses, fungi, and parasites lead to reduced fish welfare, increased mortality and added cost of production.

Gill diseases are among the health issues that causes significant mortality, economic loss, and reduced fish welfare in Norwegian salmonid aquaculture (Mowi, 2021; Sommerset et al., 2022). Gill disease and gill injuries can be caused by infectious agents, environmental factors, management operations or a combination of these, but the exact cause(s) and pathogenesis of a considerable proportion of gill disease cases are unknown (Boerlage et al., 2020; Rodger, Henry, et al., 2011). Methods to control sea lice levels have shifted from medicinal treatments to non-medicinal methods. The non-medicinal methods (NMMs) include thermal and mechanical delousing and freshwater baths (Overton et al., 2018). These new treatments for sea lice may impact gill health directly, as well as causing stress and possibly increased susceptibility to infectious disease (Gismervik et al., 2017; Nakano et al., 2014; Sommerset et al., 2021; Strand et al., 2021). During the sea phase of salmon production, regular *in sit*u cleaning of net pens is performed to prevent overgrowth and negative effects of biofouling. Laboratory studies have shown that the resulting biofouling debris may lead to gill injuries in exposed salmon (Baxter et al., 2012; Bloecher et al., 2018), though little it is known about the impact of biofouling under field conditions. Thus, more knowledge about which factors impact gill health of farmed Atlantic salmon are required to prevent gill disease and reduce the impact of disease once it occurs.

5.1.2 Gill anatomy and function

The gills (Figure 2) have a range of important functions for health and homeostasis. In addition to gas exchange, gills also play a role in osmotic and ionic regulation, acid-base regulation, and excretion of nitrogenous wastes. The filament is the basic functional unit of the gill, and the lamellae are the basic respiratory units. Gas exchange occurs at the level of the lamella where the thin barrier between water and blood and the countercurrent flow of blood and water allows for efficient removal of carbon dioxide and uptake of oxygen. Freshwater fish need to conserve and actively take up ions and get rid of excess water, while the converse is true for sea farmed salmon. Active uptake of sodium and chloride ions, and to a lesser extent calcium, magnesium, and zinc, occurs across the gill epithelium. In marine Atlantic salmon, chloride cells actively extrude chloride and sodium to keep the plasma hypoosmotic in relation to the seawater. The gill contributes to pH and acid-base regulation by regulation of respiration (respiratory compensation), exchange of acid-base equivalents and ions with the environment (metabolic compensation) and acid-base secretion across the gills. Nitrogenous waste is mainly excreted across the gills in the form of ammonia, while a small proportion is excreted as urea in the urine (Evans et al., 2005).
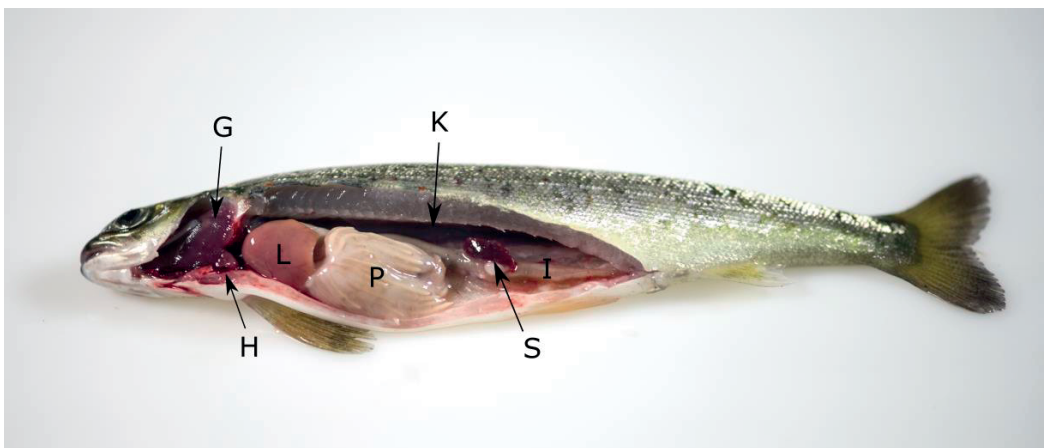
Figure 2. Anatomy of a salmonid. *The operculum and abdominal wall are removed to expose the gills (G), heart (H), liver (L), pyloric caeca (P), spleen (S), kidney (K) and posterior intestine (I). Image copyright Pharmaq Analytiq.*

Figure 3. Gill anatomy – transversal view. *Gross image of formalin-fixed gill shows a gill arch (a), gill raker (r), vessels in the gill arch (in circle), the two filaments (f) and the interbranchial septa (is). Water flows from the efferent (ef) to the afferent side (af) along the filaments.*
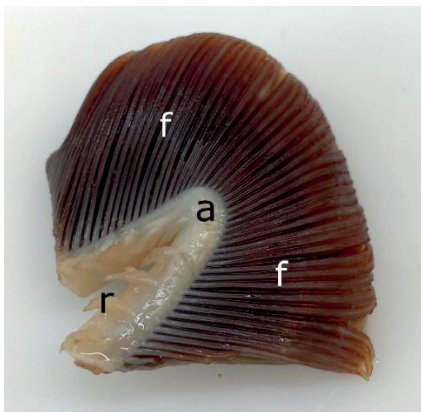
Figure 4. Gill anatomy – lateral view. *Gross image of formalin-fixed gill shows a gill arch (a), gill rakers (r) and filaments (f).*

The gills of Atlantic salmon consist of four bilateral rows of holobranchs found at each side of the pharynx. A holobranch consists of a gill arch with two rows of filaments (hemibranchs) as seen in Figure 3. The hemibranchs are connected by an interbranchial septum and protrude in a posteriolateral direction (Wilson & Laurent, 2002). Short gill rakers are found along the cranial and buccal aspect of the gill arches (Figure 4). A cartilaginous and partially ossified skeleton is found in the centre of the

arch, and branchial skeletal rays that support the filaments radiate from the arch. In addition, vessels, connective tissue, nerves, and immune cells are part of the gill arches and filaments. The surface area of the filament is increased by the presence of perpendicularly oriented rows of lamellae on the dorsal and ventral surfaces. The lamellae consist of a network of vascular spaces called sinusoids. Sinusoids are delineated by pillar cells and are covered by sheets of epithelial cells (Figure 5 and 6).
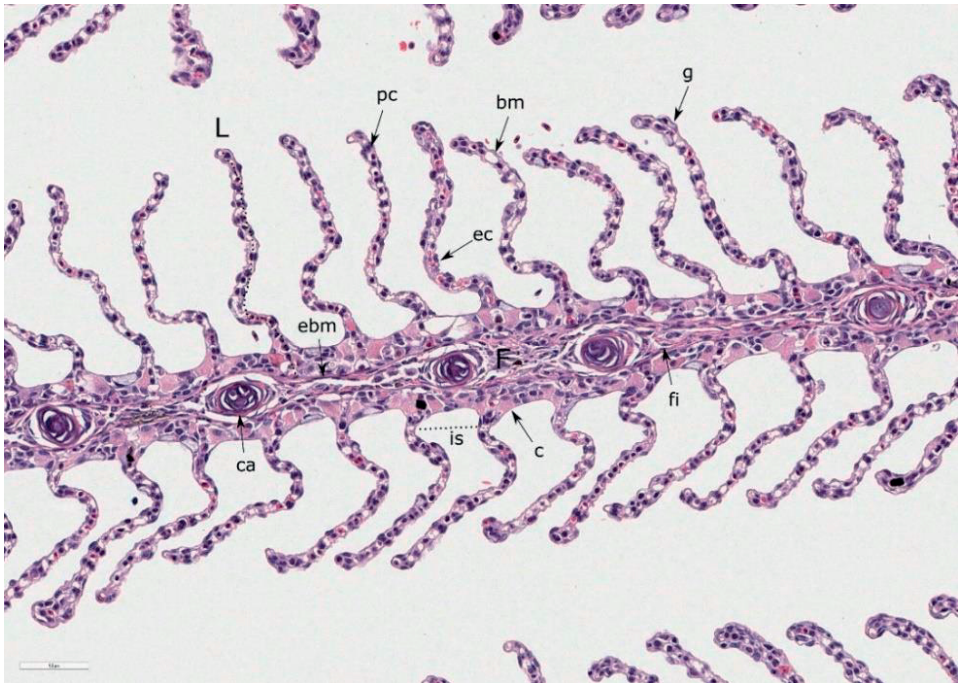
Figure 5. Microscopic gill anatomy. *Histology section of shows F - filament, L - lamella, is – interlamellar space, fi – filament interstitium, ca – filament cartilage, g – goblet cell, pc – pillar cell, ec – epithelial (pavement) cell, c – chloride cell, bm – basement membrane, ebm – basement membrane of the interlamellar epithelium.*

Pillar cells are modified endothelial cells that envelop collagen columns connecting the opposing sides of the lamellar vascular space. These collagen columns are continuous with the basement membrane and help maintain the structural integrity of the lamella. The lamellar epithelium primarily consists of squamous to cuboidal epithelial cells called pavement cells. The lamellar epithelium shares the basement membrane with the pillar cells (Evans et al., 2005; Wilson & Laurent, 2002). The epithelium covering the filament usually consists of more than 3 cell layers, whilst the cell layer of normal lamella is 1 to 3 cells in thickness. In addition to squamous

epithelial cells, mitochondria-rich cells (chloride cells), accessory cells, and goblet (mucus) cells are frequently found within the filament epithelium (Figure 5). Variable numbers of lymphocytes, macrophages, eosinophilic granular cells, neuroepithelial cells and rodlet cells are found in the filament interstitium (Speare & Ferguson, 2006).
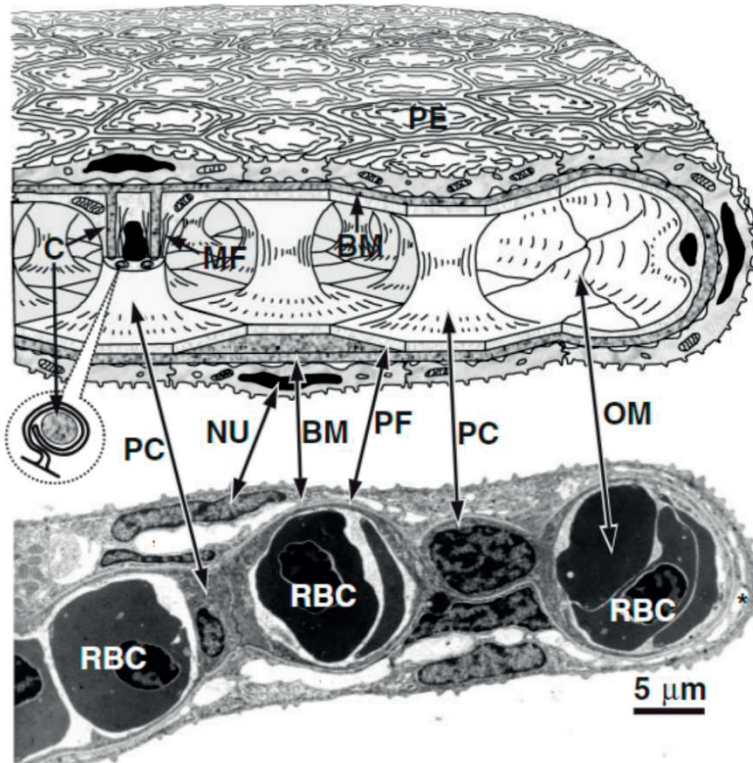
Figure 6. Schematic and transmission electron micrograph of a lamella. Pillar cells (PC) have cytoplasmic flanges (PF) that line lamellar sinusoids and contain microfilaments (MF) that may be involved in pillar cell contraction. In the vascular spaces are red blood cells (RBC). The pillar cells envelop collagen columns (C) that are connected to the basement membrane (BM) that is shared with the lamellar epithelium (PE). The nucleus of the epithelial cell (NU) is flattened. The outer marginal channel (OM) is lined internally by pillar cells, with epithelial cells covering the external surface. Image reproduced with permission from Olson (2002).

Two or three circulatory systems have been described in the gills depending on what the authors define as separate systems (Evans et al., 2005; Olson, 2002). The respiratory arterioarterial pathway consist of afferent and efferent branchial arteries close to the filament trailing and leading edges, respectively, and the interposed lamellar circulation. The non-respiratory interlamellar and nutrient vascular systems are post-lamellar circulation found in the filaments. Intraepithelial immune tissues are found in the interbranchial septa (proximal interbranchial lymphoid tissue), along the trailing edge of the filaments (distal interbranchial lymphoid tissue) and along the posteriolateral edge of the gill arches and base of the filaments (amphibranchial lymphoid tissue) (Bjørgen & Koppang, 2021; Dalum et al., 2021). Laterally the gills are covered and protected by a bony operculum. Malformation or damage to this structure predispose the gill to injury.

5.1.3 Concepts of disease, aetiology, and pathogens

The gills can be affected by a wide range of infectious and non-infectious diseases and insults. A disease can be defined as "any deviation from or interruption of the normal structure or function of a part, organ, or system of the body manifested by characteristic symptoms and signs; the etiology, pathology, and prognosis may be known or unknown" (Dorland's Illustrated Medical Dictionary, 2020). Further Myers and McGavin (2007) state that "Disease is not just illness or sickness but includes any departure from normal form (lesions) and function, whether it is clinically apparent or not". The aetiology of a disease is the cause or causes of the disease. Different concepts and models of disease causation exist. The main model of causation used by epidemiologists is the component-cause model (causal pie model) developed by Rothman (Rothman & Greenland, 2005; Vineis & Kriebel, 2006). In this model a necessary cause is one which is necessary for disease to occur, and in its absence disease cannot occur. For instance, *N. perurans* is a necessary cause of amoebic gill disease. A sufficient cause will always lead to disease if present. However, because relatively few exposures (infectious agents, genetic variants, environmental factors) are sufficient to cause disease alone, several factors can combine to become a sufficient cause. A component-cause is then one of several factors that together is a sufficient cause. E.g., presence of *N. perurans*, higher water temperatures and high salinity can all be considered component-causes for amoebic gill disease. Dohoo et al. (2014a) defines a cause as any factor that leads to a change in the severity or frequency of the outcome (i.e., disease). However, in this thesis a cause of disease will

refer to one or more necessary causes for which a biological mechanism of disease is known or plausible, while a risk factor for a disease is any factor that increase the likelihood of the disease in the absence of a direct mechanistic cause (Dohoo et al., 2014a; Shader, 2019).

Traditionally, microorganisms have been classified as pathogenic and non-pathogenic. Primary pathogens were defined as those that cause disease in a healthy host, while opportunistic pathogens usually require an injured or immunocompromised host to cause disease. In contrast, pure saprophytes or non-pathogenic microorganisms are unable to grow in healthy living tissues (Méthot & Alizon, 2014). A facultative pathogen can survive and proliferate in an environmental reservoir such as water or soil but can potentially cause disease when encountering a susceptible host. Obligate pathogens are obligate parasites and can only proliferate in a receptive host animal. The normal flora or microbiota are the microorganisms that normally inhabit the external or internal surfaces of animals, like skin, gills, or gastrointestinal tract. In addition to the permanently colonizing species, environmental microorganisms only temporarily colonizing the host form a transient microbiota. The microbiota is generally considered to have a positive impact on health and are necessary for normal development of the immune system (Fiebiger et al., 2016; Khan et al., 2019). However, microorganisms of the normal flora can in some cases become opportunistic pathogens causing disease in a susceptible host (Slater, 2007).

Infection or colonization of host tissues or surfaces with a pathogen does not necessarily result in disease, and subclinical or latent infection or healthy carrier states are common in human and animal populations. Co-infections, infections with more than one pathogen, are also commonplace (Kotob et al., 2016). A co-infection is defined as occurring concurrently with the initial infection with a given agent. Secondary infections are infections following a previous infection (Feldman & Anderson, 2021). The pathogenicity of a microbe refers to whether infection with the organism causes disease or not. Virulence refers to the relative capacity of a microorganism to cause damage in a host (Casadevall & Pirofski, 2003). With increasing knowledge about the pathogenesis of infectious diseases it has become clear that pathogenicity and virulence of microorganisms not only depends on properties inherent to the infectious agent. Virulence and pathogenicity of a microbe are also affected by the host and environment and interactions between all three factors. Méthot and Alizon (2014) argue that pathogen virulence can be viewed as

result of the interaction between 2 genomes (host and parasite) and the environment (GxGxE interaction), where the number of genomes could be increased to account for co-infections. Similarly, within epidemiology the epidemiologic triad (Figure 7) is a model of infectious disease used to illustrate the interplay between the pathogen, environment and host (Johnson-Walker & Kaneene, 2018).
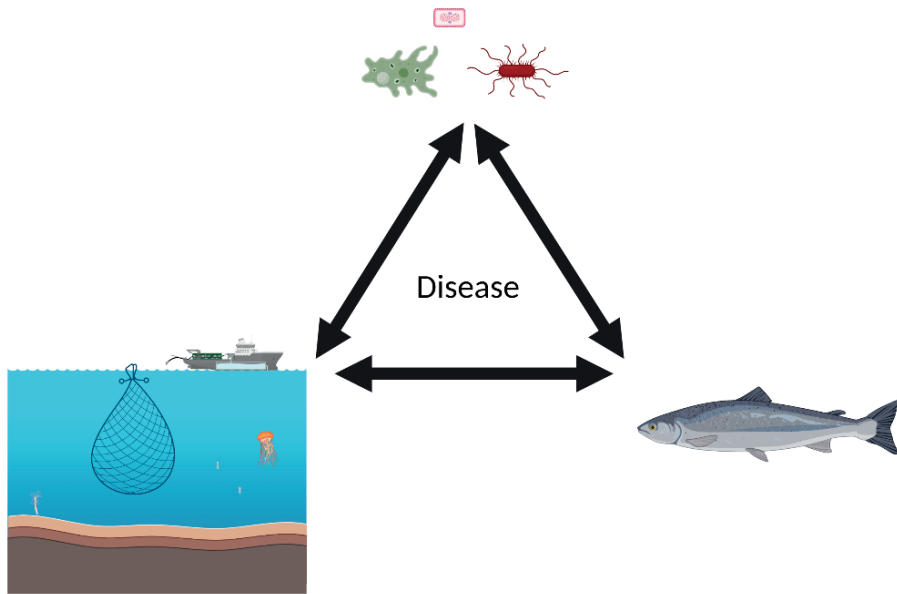
Figure 7. The epidemiologic triad and infectious gill disease. The triad depicts the host (Atlantic salmon), the environment (sea cages and surrounding waters) and pathogens. Using a wide definition of environmental factors managerial factors such as net-management practices and delousing regimens can be considered a part of the environment (Figure created in BioRender.com).

5.1.4   Terminology and diagnosis of gill disease

In veterinary medicine, a clinical diagnosis of disease is generally made based on case history, clinical signs, and physical examination of an individual animal. Additionally, samples for laboratory analysis may be submitted to detect pathogens or measure levels of metabolites in the blood or other body fluids. Imaging tools such as x-rays or

ultrasonography can be used for examination of the skeleton and internal organs. In salmonid health management it is generally not the individual fish, but the population of fish in a pen that is the unit of interest. Clinical signs of disease in salmonids are frequently non-specific, the behaviour of fish in sea cages can be difficult to assess and detailed physical examination of live fish is generally not feasible. For these reasons, diagnosing diseases in fish is largely reliant upon observations of abnormal behaviour, increased mortality, and reduced appetite within a group of fish combined with necropsy and gross examination of euthanized and dead individuals. Polymerase chain reaction (PCR) analysis is used extensively for pathogen monitoring and detection, and histopathology is used for microscopic assessment of tissue injuries and lesions associated with infectious or non-infectious diseases. Bacterial culture and new molecular methods like next-generation sequencing (NGS) are also increasingly being used for diagnostic investigation of suspected bacterial disease and new disease entities, respectively. Microscopy of fresh tissues or smears on-site can be helpful to identify infectious agents. In Norway gross gill scoring is primarily used to monitor the prevalence and severity of AGD to help determine whether intervention is necessary (Hytterod et al., 2018). Water sampling for assessment of zoo- and phytoplankton levels and species classification are commonly performed in Canada, Scotland, and Ireland but this is not regularly or systematically performed at Norwegian sea sites.

Each diagnostic method has advantages and limitations, and often a combination of methods will yield the best result. PCR-analysis is the superior method for determining whether fish are infected or colonized with one or more specific microbes. The method cannot be used to diagnose non-infectious disease and injury, nor new or emerging infectious agents with unknown genetic sequences. Histopathology is used to determine whether morphological changes consistent with disease are present in fish tissues. A disease diagnosis and aetiology are provided if tissue lesions are characteristic of a specific disease, alternatively likely or possible causes of disease are listed. Histopathology allows for characterization of lesions and grading of lesion severity. Further, with the help of *in-situ* hybridization (ISH) and immunohistochemistry the location of pathogens within tissues and their relationship to tissue lesions can be observed and described.

The terminology of gill disease in salmonids is slightly confusing as the growing knowledge in the field is not always reflected in terms used to describe gill diseases and injuries. Proliferative gill inflammation (PGI) was first suggested to be a distinct,

yet multifactorial gill disease of farmed Atlantic salmon by Kvellestad et al. (2005). Histologic criteria for this condition were co-localization of four types of pathology, namely inflammation, epithelial hyperplasia, circulatory disturbances, and cell death of lamellar epithelium. In addition, the term PGI was initially suggested to be used only in cases with clinical signs of gill disease, such as increased mortality (Kvellestad, 2013), but in some studies this diagnosis is based on histopathology results alone (Steinum et al., 2010). Proliferative gill disease (PGD) is a non-specific term used in the UK when proliferative gill lesions are identified by gross examination but have also been used by researchers when hyperplasia of lamellar epithelium was observed on microscopic examination (Herrero et al., 2018; Nylund et al., 2008). However, neither PGD nor PGI are specific diseases with established or specific aetiologies and may have been defined and understood differently among different researchers.

Epitheliocystis is a general term used to describe any disease associated with the presence of intracytoplasmic bacterial cysts (epitheliocysts) in the gill or skin epithelium of different fish species (Blandford et al., 2018; Hoffman et al., 1969). Some researchers have also used the term interchangeably with proliferative gill inflammation (PGI), suggested using presence of epitheliocysts as criteria for the PGI-diagnosis or diagnosed PGI based on clinical signs alone (Grøntvedt et al., 2006; Gunnarsson et al., 2017). Complex gill disease (CGD) was initially reported as a term used by fish health personnel in Scotland as a catch-all term for gill disease presumed to be caused by more than one infectious agent and/or environmental factor (Herrero et al., 2018). The term is reported to encompass cases of PGD and PGI and may also include cases of AGD (Herrero et al., 2018). A wide range of gill lesions and microorganisms, and different lesion and pathogen combinations can qualify for a diagnosis of CGD (Noguera et al., 2019). This underscores that CGD, as PGI and PGD, is not a specific disease likely to have a common aetiology or aetiologies and pathogenesis across cases and geographical locations.

5.1.5 Gill disease of Norwegian farmed Atlantic salmon

A summary of the most important putative gill pathogens, environmental and managerial factors contributing to gill disease and injury in Norwegian sea farmed Atlantic salmon follow in the next sections. The descriptions are not exhaustive and are limited to the infectious and non-infectious diseases most relevant for the current study. Pathogens infecting gills, but primarily or only causing pathology in other organ systems may have been excluded.

5.1.5.1 Infectious gill disease

Salmon gill poxvirus

Salmon gill poxvirus (SGPV) was first detected in gills of sea farmed Atlantic salmon suffering from proliferative gill disease in 2006 (Nylund et al., 2008). Later studies confirmed that the virus had been present and caused disease in Norwegian farmed Atlantic salmon as early as 1995 (Gjessing et al., 2015). SGPV is widely distributed in wild salmon in Norway. The virus was detected in salmon caught at sea and in rivers from Hordaland County in the south to former Finnmark County in the north (Garseth et al., 2018; Kambestad, 2019). Horizontal transmission has been shown in a cohabitation study, but there is currently no evidence for vertical transmission (Wiik-Nielsen et al., 2017). Introduction of the virus into freshwater facilities may occur through virus-contaminated intake water or through introduction of infected fish.

Virus infection can lead to salmon gill poxvirus disease (SGPVD) and high, acute mortality (Gjessing et al., 2015). Clinical signs of SGPVD include lethargy, respiratory distress, and mortality (Gjessing et al., 2018; Gjessing et al., 2015; Nylund et al., 2008). Gross lesions reported are minimal and includes swollen and slightly pale gills (Gjessing et al., 2015), although necrotic patches on the gills are described in one study (Nylund et al., 2008). The characteristic acute histological lesion is lamellar epithelial cell apoptosis, apparent as swollen, cuboidal, and detaching (budding) epithelial cells with margination of nuclear chromatin (Figure 8a). In addition, epithelial hypertrophy, collapse, and adhesion (synechia) of denuded lamella and degenerated chloride cells are described. Lamellar epithelial proliferation,

displacement and degeneration of chloride cells dominate in the later regenerative phase (Gjessing et al., 2015). SGPVD primarily appears to be problem in juvenile fish in freshwater facilities, including fish smaller than 0.5 grams (fry) (Gjessing et al., 2018). The role of SGPV in outbreaks of gill disease during the sea phase of production is less clear but the virus has been detected in apoptotic gill epithelium of sea farmed salmon with proliferative and complex gill pathology (Gjessing et al., 2021; Gjessing et al., 2017; Nylund et al., 2008).

Desmozoon lepeophtherii (*Paranucleospora theridion*)

Desmozoon lepeophtherii (synonym *Paranucleospora theridion)* is a fungal organism belonging to the phylum Microsporidia. The agent infects both sea lice (*Caligus elongatus* and *Lepeophtheirus salmonis)* and Atlantic salmon, sea trout, rainbow trout and various species of wrasse (Nylund et al., 2010; Steigen et al., 2018). It was first detected in salmon and *Lepeophtheirus salmonis* in Scotland in 2000 (Freeman, 2002). *D. lepeophtherii* was subsequently characterized and found to be identical with the microsporidian *Paranucleospora theridion* detected in Norwegian Atlantic salmon and sea lice (Freeman et al., 2003; Freeman & Sommerville, 2009, 2011; Nylund et al., 2010). *D. lepeophtherii* infection is commonly found in sea farmed Atlantic salmon in southern and western Norway. Infected fish populations do not clear the infection but appear to become persistently infected (Gunnarsson et al., 2017; Nylund et al., 2011; Steinum et al., 2015; Steinum et al., 2010; Sveen et al., 2012). Infections prior to sea transfer are rare but have occurred at sites where seawater has been used to increase salinity to facilitate smoltification (Nylund et al., 2011).

How the infection spreads from sea lice to sea lice and the route of infection for Atlantic salmon remains unclear. Vertical transmission in sea lice has been suggested, but not confirmed (Nylund et al., 2010; Økland, 2012). Field studies indicate that sea lice do not have to be present in the salmon population for fish to become infected, and waterborne spores previously released by sea lice has been suggested as a source of infection (Herrero, Rodger, et al., 2022; Sveen et al., 2012). Experimental infection of Atlantic salmon has not been reported in the scientific literature. Attempts to infect fish or fish cell lines with spores isolated from salmon lice have so far been unsuccessful (Freeman, 2002; Herrero-Fernández, 2019; Smørås, 2014). After infection the organism is detected in fish gills prior to systemic infection. Release of spores may occur from gills, skin, and gut epithelium (Nylund et al., 2010; Sveen et

al., 2012; Weli et al., 2017). Two developmental cycles occur in salmon. These lead to development of auto-infective and intracytoplasmic spores (~ 1 µm in diameter) in phagocytes, epithelial cells, and endothelial cells or to development of environmental and intranuclear spores (~ 2,5 x 2,0 µm) in epithelial cells only (Nylund et al., 2010). In addition, round, 4–6 µm in diameter presporogonic stages may be observed in the cytoplasm of gill epithelial and endothelial cells (Herrero, Palenzuela, et al., 2022).

Gross gill lesions described during clinical outbreaks associated with *D. lepeophtherii* are pale and swollen gills (Hamadi, 2011; Nylund et al., 2010). Microscopic lesions reported to be associated with *D. lepeophtherii* (Figure 8b) include ballooning degenerative cells containing pigmented material, lamellar epithelial hyperplasia and hypertrophy, necrosis in hyperplastic lesions, subepithelial inflammation, and necrosis (Gjessing et al., 2021; Herrero, Palenzuela, et al., 2022; Matthews et al., 2013; Nylund et al., 2010; Weli et al., 2017). Inflammation have also been reported in other organs (Nylund et al., 2010). Infection without associated gill pathology or clinical gill disease is common (Downes et al., 2018; Herrero, Rodger, et al., 2022; Nylund et al., 2011; Steinum et al., 2015; Steinum et al., 2010). Fungal spores may be observed in lesions, but because these and other stages of the organism are very small and may be single or in small clusters, they can be difficult to observe in HE-stained tissue sections (Herrero, Palenzuela, et al., 2022; Matthews et al., 2013). Immunohistochemistry and *in situ* hybridization have higher sensitivity for detection of the organism in tissue sections, though calcofluor white and Gram staining also improve detection compared to HE-stained sections (Herrero et al., 2019; Herrero, Palenzuela, et al., 2022). However, PCR-analysis is primarily used to diagnose infection and assess pathogen load.

Candidatus Branchiomonas cysticola and other bacteria associated with epitheliocysts in the gills

Intracytoplasmic and intraepithelial bacteria (Figure 8c) have been observed in the gills of Norwegian farmed Atlantic salmon since the early 90's (Nylund et al., 1998). Bacteria from the order *Chlamydiales* were initially identified as the epitheliocyst-forming bacteria in farmed Atlantic salmon. *Candidatus* Piscichlamydia salmonis was characterized in Norwegian and Irish Atlantic salmon samples from 1999 and 2000 (Draghi et al., 2004), followed by characterization of *Candidatus* Clavochlamydia salmonicola in Atlantic salmon from Norwegian freshwater facilities and wild trout

(*Salmo trutta*) in 2005-2006 (Karlsen et al., 2008). Description and characterization of the betaproteobacterium *Candidatus* Branchiomonas cysticola (*Ca.* B. cysticola) and the chlamydia *Candidatus* Syngnamydia salmonis was reported in 2012, 2013 and 2015, respectively (Mitchell et al., 2013; Nylund et al., 2015; Toenshoff et al., 2012). Detection of a new epitheliocyst-forming betaproteobacterium was reported in 2015, but characterization, information about prevalence and importance of this bacterium is currently lacking (Wiik-Nielsen et al., 2015). *Candidatus* Clavochlamydia salmonicola infections lead to formation of epitheliocysts but are not associated with clinical gill disease or any other types of gill pathology (Mitchell et al., 2010; Quezada-Rodriguez et al., 2022). *Candidatus* Clavochlamydia salmonicola infections resolve after sea transfer of affected fish (Mitchell et al., 2010). *Candidatus* Syngnamydia salmonis is frequently found within amoeba. The bacterium itself do not appear to be associated with severe gill disease (Nylund et al., 2015). *Candidatus* Piscichlamydia salmonis was initially found to be associated with gill disease (proliferative gill inflammation) (Steinum et al., 2010; Steinum et al., 2009). Later studies failed to find an association between *Ca.* Piscichlamydia salmonis and gill lesions suggesting that this agent may not be causing gill disease (Gjessing et al., 2021; Gjessing et al., 2019).

The gill microbiota of Atlantic salmon is dominated by Proteobacteria (Brown et al., 2021; Lorgen-Ritchie et al., 2022; Slinger et al., 2021), and in some studies bacteria of the taxon *Candidatus* Branchiomonas is the most abundant bacteria in the gills of Atlantic salmon and rainbow trout (Brown et al., 2021; Brown et al., 2019). *Ca.* B. cysticola has been found to be the most common epitheliocyst forming agent in farmed Atlantic salmon in Norway and Ireland (Gjessing et al., 2021; Mitchell et al., 2013; Toenshoff et al., 2012). Infections are found in both freshwater and seawater and the bacterium is nearly ubiquitous in sea farmed salmon in Northern Europe (Downes et al., 2018; Gunnarsson et al., 2017; Herrero-Fernández, 2019; Steinum et al., 2010). A high prevalence of infection has been found in wild Norwegian Atlantic salmon caught in rivers and at sea (Kambestad, 2019).

In a co-habitation study exposed fish were infected with *Ca.* B. cysticola, SGPV and *Ca.* Piscichlamydia salmonis, confirming horizontal transmission between fish. Infected cohabitants developed mild to moderate epithelial hyperplasia and subepithelial inflammation, but signs of clinical disease and mortality was not observed (Wiik-Nielsen et al., 2017). *Ca.* B. cysticola can infect gill tissues without forming the characteristic intracellular cysts (Gjessing et al., 2021; Gjessing et al., 2019). Histologic lesions associated with *Ca.* B. cysticola infection in fish from sites with gill

disease include lamellar epithelial hyperplasia, subepithelial inflammation, pustules, and necrosis (Gjessing et al., 2021; Gjessing et al., 2019). In addition, subepithelial neutrophilic inflammation presumed to be associated with *Ca.* B. cysticola infection has been observed at multiple fresh- and seawater sites in Norway recently (E. Thoen personal communication). However, whether *Ca.* B. cysticola cause gill disease or whether it is an important driver of gill pathology in sea farmed salmon remains controversial.

Amoebic gill disease (AGD)

Amoebic gill disease (AGD) was first described as a problem in farmed salmonids in the 1980s in Tasmania (Munday, 1986, cited by Oldham et al., 2016), but has since been reported in all major salmon producing areas including Norway, UK, North America, and Chile (Bustos et al., 2011; Kent et al., 1988; Rodger & McArdle, 1996; Steinum et al., 2008; Young et al., 2008). The first outbreak of AGD in Norway was reported in 2006, and since 2012 the disease has been endemic in Norwegian salmonid mariculture (Mo et al., 2015; Sommerset et al., 2021; Steinum et al., 2008). Amoebic gill disease is currently considered the most important pathogen associated with gill disease in Norwegian marine salmon farming (Powell et al., 2015; Sommerset et al., 2022). AGD is caused by the free-living (amphizoic), facultative ectoparasite *Neoparamoeba perurans* (Crosbie et al., 2012; Young et al., 2007).

The environmental reservoir of *N. perurans* is unknown, but studies suggest the parasite is a cosmopolitan organism. It has been detected in water samples from farms with affected salmon, in net biofouling, sediments, sea lice, plankton and wild fish (Bridle et al., 2010; Crosbie et al., 2005; Douglas-Helders et al., 2003; Hellebo et al., 2017; Nowak et al., 2010; Tan et al., 2002; Young et al., 2008). However, there is no evidence that the water column, wild fish, salmon ectoparasites, biofouling or sediments are significant reservoirs of infection (Hellebo et al., 2017; Oldham et al., 2016). Outbreaks of AGD have occurred when salmonid mariculture was initiated in high salinity seawater in new areas with locally cultivated fish, suggesting a local environmental source of amoeba (Mouton et al., 2014). Cleaner fish used for sea lice control can become infected, develop amoebic gill disease and may be a source of infection for farmed salmon (Haugland et al., 2017; Karlsbakk et al., 2013). Wild fish living in the vicinity of a sea farm may become infected during AGD outbreaks and these have been suggested as a source of reinfection after treatment (Hellebo et al.,

2017). Amoeba may be transported from one farm to another by currents free in the seawater or associated with plankton, after infecting mobile fish species (pollock, mackerel) or on escaped salmon. It has recently been shown that amoeba can survive freshwater treatment and may reinfect salmon if used treatment water is released back into the sea (Taylor et al., 2021).

Disease outbreaks have been associated with high or higher than normal water temperatures (>12°C) and high salinity (>32‰) (Clark & Nowak, 1999; Jones & Price, 2022; Munday et al., 2001; Oldham et al., 2016). However, clinical disease has been observed at temperatures lower than 10°C (Rodger, 2014). Higher pathogen load and more severe pathology was found in fish experimentally infected with *N. perurans* at 15°C versus 10°C (Benedicenti et al., 2019). In Norway *N. perurans* infection and amoebic gill disease occur in the autumn during or after the period with the highest water temperatures of the year (Mo et al., 2015; Persson & Nygaard, 2014). It has been reported that stocking density and biomass, oxygen levels (hypoxia), number of net-changing events, heavily fouled nets and low water exchange can impact disease prevalence and outcomes (Clark & Nowak, 1999; Crosbie et al., 2010; Douglas-Helders et al., 2004; Oldham et al., 2020; Rodger, 2014).

Clinical signs reported in fish with AGD include lethargy, anorexia, increased ventilation rate, fish gathering at the water surface and ultimately death (Kent et al., 1988; Munday et al., 1990, cited by Oldham et al., 2016). White to grey, raised, focal or multifocal patches with excess mucous on the gills are characteristic gross findings, though these may not always be present and are preceded by histological changes (Adams et al., 2004; Adams & Nowak, 2001; Adams & Nowak, 2003; Clark & Nowak, 1999). Gross lesions have been reported to be most numerous in the dorsal, ventral, and proximal part of the gill arches and on the second gill arch (Hytterod et al., 2018). Patches observed grossly correspond to microscopic lesions (Figure 8d) of marked segmental epithelial hyperplasia, goblet cell hyperplasia, lamellar fusion, and interlamellar cysts or vesicles in the hyperplastic epithelium (Adams et al., 2004; Zilberg & Munday, 2000). Other histopathological findings include loss of chloride cells, accumulation/infiltration of neutrophilic granulocytes, macrophages, and lymphocytes in the central venous sinus, interstitium of the filament and in hyperplastic tissue. Leukocytes, debris, and parasites can be found in cysts or vesicles (Adams & Nowak, 2001; Chang et al., 2019). Sequential sampling has shown that histological lesions progress from initial hypertrophy, desquamation, mild epithelial hyperplasia and oedema to accumulation and infiltration of leukocytes,

sloughing/necrosis, spongiosis and pronounced hyperplasia (Adams & Nowak, 2003). Electron microscopy studies revealed that amoeba penetrate lamellar epithelium and leave indentations and fenestrations, disrupting normal surface microridges and leading to exfoliation of affected cells (Lovy et al., 2007; Roubal et al., 1989; Wiik-Nielsen et al., 2016).

Amoebic trophozoites are primarily associated with hyperplastic lesions. They are 13.6-24.1 μm in diameter and up to 37 μm long, oval to irregularly shaped with granular and vacuolated basophilic cytoplasm, a juxtanuclear parasome(s) and an amphophilic round to horseshoe-shaped karyosome in the nucleus (Karlsbakk et al., 2013). The parasome is an obligate eukaryotic endosymbiont, an intracellular organism in a symbiotic relationship with the amoeba. These organisms belong to the genus *Perkinsus* spp. and are flagellated protists (class Kinetoplastea) (Nowak & Archibald, 2018; Tanifuji et al., 2017). Amoeba from the natural environment is also associated with bacteria which have been found to multiply inside the amoeba. Both amoebae-associated bacteria, the *Perkinsus* spp. endosymbiont and other bacterial species and commensals have been suggested to play a role in pathogenesis of AGD (Nowak & Archibald, 2018; Slinger et al., 2020; Taylor et al., 2021).

Freshwater and hydrogen peroxide baths are currently used for treatment of AGD in commercial salmon farming (Martinsen et al., 2018; Powell et al., 2015). As reviewed by Oldham et al. (2016) a wide range of other treatments have been tested. Several studies have shown that resistance to AGD infection and the extent of gill pathology after challenge is heritable and possible candidate genes conferring resistance have been identified. Breeding programs to develop more resistant salmon is also underway in several parts of the world (Aslam et al., 2020; Boison et al., 2019; Gjerde et al., 2019; Kube et al., 2012; Lillehammer et al., 2019; Taylor et al., 2007).
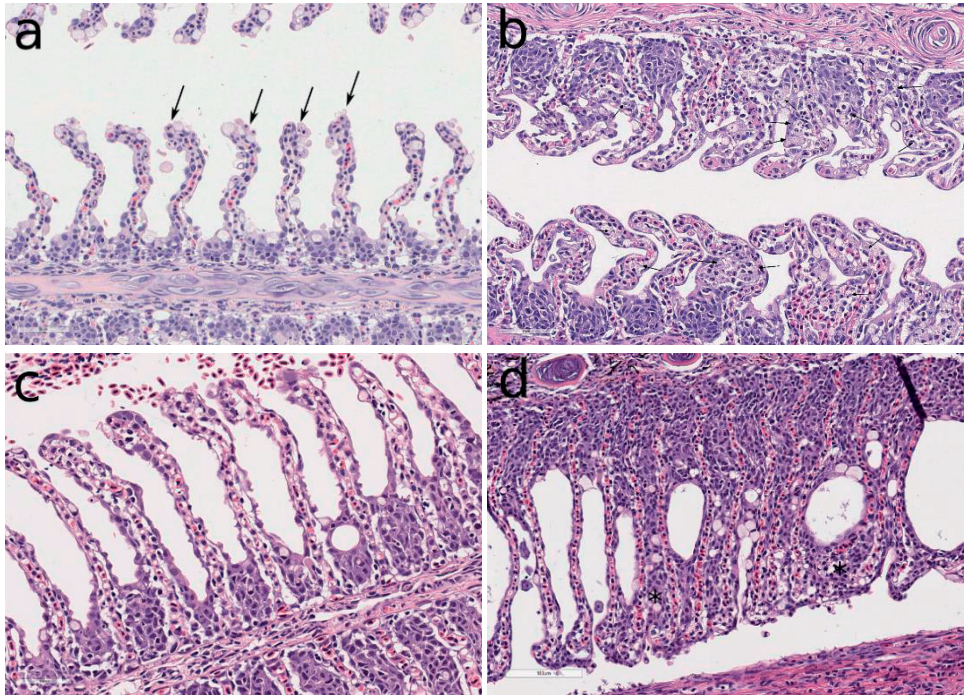
Figure 8. Infectious agents and associated pathology. a) SGPV, epithelial cell apoptosis, swollen cells with condensation and margination of nuclear chromatin (arrows), b) Lesions reported to be associated with D. lepeophtherii. Subepithelial ballooning cells containing pigmented material, infiltration of inflammatory cells and necrosis (arrows), c) Epitheliocyst, intracellular bacteria (possibly Candidatus Branchiomonas cysticola), d) N. perurans, note hyperplasia and fusion of surrounding lamella (*).

Miscellaneous infectious agents

Atlantic salmon paramyxovirus (ASPV) was first isolated from fish with gill disease and was initially suspected to contribute to development of disease (Kvellestad et al., 2003; Kvellestad et al., 2005). However, a challenge experiment showed that viral infection was not associated with pathology or mortality (Fridell, 2003; Fridell et al., 2004). In later field studies ASPV was rarely detected in salmon diagnosed with proliferative gill disease in Norway (Nylund et al., 2008; Nylund et al., 2011; Steinum et al., 2010).

Tenacibaculum spp. are Gram-negative, filamentous bacteria (Figure 9a) in the Flavobacteriaceae family, associated with ulcerative and necrotizing diseases (tenacibaculosis) in many marine fish species (Avendaño-Herrera et al., 2006; Bridel et al., 2018; Frisch et al., 2018). *T. maritimum* has been isolated from healthy and diseased wild and cultured fish species and is widely present in marine environment (Fringuelli et al., 2012; Rud et al., 2017; Wynne et al., 2020). Risk factors reported for tenacibaculosis include chemical or physical disruption of fish surfaces, high or low water temperatures (depending on *Tenacibaculum* species), high salinities and elevated ammonia. Disease has been induced experimentally in Atlantic salmon smolts without prestress, disruption of epithelial barriers or coinfection with other pathogens (Avendaño-Herrera et al., 2006; Frisch et al., 2018; Mitchell & Rodger, 2011; Powell et al., 2004). Gill disease associated with *T. maritimum* in Norwegian Atlantic salmon without concurrent skin or mouth lesions were described in 12 sea farms Western Norway in 2017 and 2018. The outbreaks of necrotizing gill disease associated with *T. maritimum* occurred in fish that also had gill lesions ascribed to other pathogens (R. Johansen personal communication).

Ichthyobodo spp. are opportunistic, ectoparasitic flagellates (Figure 9b) that infect a range of fish species across the world (Urawa et al., 1998). In salmon farming disease caused by infection (ichthyobodosis) is primarily a problem in the freshwater phase, and outbreaks of disease in seawater was first reported in the 1970's (Ellis & Wootten, 1978; Poppe & Håstein, 1982). *Ichthyobodo necator* sensu stricto is found in freshwater and primarily infects the skin and to a lesser extent the gills. The euryhaline *Ichthyobodo salmonis* sp. n. is found in both freshwater and seawater and is primarily found in the gills (Isaksen et al., 2010; Isakset et al., 2011; Todal et al., 2004). Trichodinids are mobile peritrich ciliates (Figure 9c) that can attach temporarily to fish skin or gills while feeding. Most often these behave as commensals and feed on waterborne and fish surface particles without causing disease. However, they may increase in numbers and cause disease and pathology in debilitated hosts (Bruno et al., 2006). Flatworms (trematodes and monogeneans) and arthropods (crustaceans) (Figure 9d) are sporadically found in gills of farmed Atlantic salmon and may lead to tissue reactions and injuries.
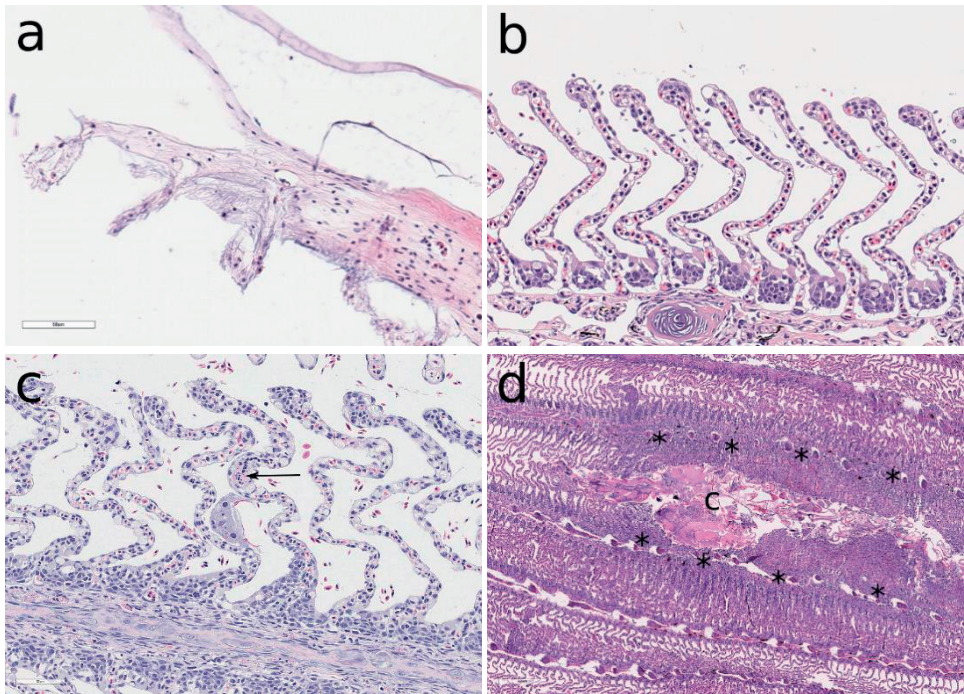
Figure 9. Infectious agents observed in gill tissue. *a) Filamentous bacteria in sea farmed salmon, most likely Tenacibaculum spp., b) Ichthyobodo spp., likely I. salmonis as fish was kept in seawater, c) Trichodina spp., also note thrombus in lamella (arrow), d) Large crustacean (C) with inflammation in opposing filaments (*), epithelial hyperplasia, haemorrhage, and loss of lamella.*

Gill disease as part of systemic infection

Gill lesions can be found in fish with systemic infection and disease caused by bacteria and other pathogens even if the gill is not the primary or only organ system affected. Intravascular bacteria and associated lamellar thrombosis, necrosis and inflammation can be a feature of bacterial diseases like bacterial kidney disease, pasteurellosis, furunculosis and mycobacteriosis (Legård & Strøm, 2020; Poppe & Ferguson, 2006) (Figure 10a-c). P*asteurella* sp. infection was first detected Norwegian Atlantic salmon diagnosed with the disease varracalbmi in 1989. Panophthalmitis, skin ulcerations, septicaemia, and pyogranulomatous inflammation in internal organs, pseudobranch and gills was found in naturally infected fish, and disease was reproduced in challenge trials (Valheim et al., 2000). Pasteurellosis, caused by the currently unofficially named *Pasteurella atlantica* genomvar

salmonicida, became endemic in Southwestern and Western Norway in 2018 and is currently an important cause of disease outbreaks and mortality in these areas (Gulla et al., 2020; Legård & Strøm, 2020; Sommerset et al., 2022). Gill lesions are sporadically found in fish with different systemic fungal and parasitic infections (Figure 10d). Lastly, gills are a port of entry or site of proliferation for several bacterial and viral pathogens that primarily cause pathology in other organ systems (Herath et al., 2016; Løvoll et al., 2009; Weli et al., 2013; Aamelfot et al., 2016; Aamelfot et al., 2012).
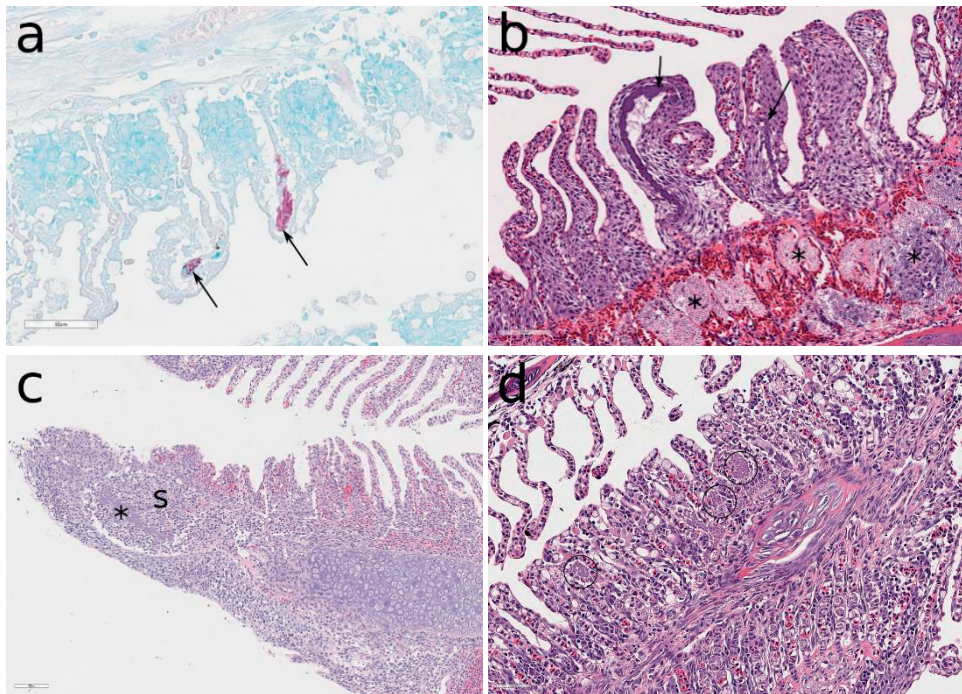
Figure 10. Systemic infection and gill disease*. a) Mycobacteriosis. Acid-fast mycobacteria in lamellar sinusoids (arrows), Ziehl Neelson stain. Infection with Mycobacterium salmoniphilum was confirmed by bacterial culture and PCR-analysis. b) Pasteurellosis. Large amounts of rod-shaped bacteria are found in the filament (*) and in the lamellar sinusoids (arrows). c) Furunculosis. Dense aggregates of rod-shaped bacteria (*) in a focus of suppuration (s) within the filament. Aeromonas salmonicida subspecies salmonicida infection was confirmed by PCR-analysis. d) Spironucleosis. Abundant pear-shaped flagellate parasites are found in the circulation and within hyperplastic lamellar epithelium (circles). Later PCR-analysis of tissue from the site*

confirmed infection with Spironucleus salmonicidae, while results for Spironucleus barkhanus were negative.

5.1.5.2   Non-infectious gill disease

Phytoplankton

Harmful algal blooms (HABs) affecting Norwegian salmonid mariculture appear to be relatively rare and sporadic events. HABs have been reported from Oslofjorden to Vestfjorden and can lead to high mortalities and losses when they occur (Karlson et al., 2021). A wide range of potentially harmful phytoplankton species can be found in Norwegian waters. Amongst these are *Chrysochromulina*, *Prymnesium*, dinoflagellate genera *Akashiwo*, *Karenia, Alexandrium* and *Karlodinium*, dictyochophyte genera *Dictyocha* (including *Octonaria*), *Pseudochattonella (Verrucophora)* and diatoms *(Bacillariophyceae)* like *Chaetocerous* spp. (Johnsen & Lein, 1989; Karlson et al., 2021; Rodger, Henry, et al., 2011). HABs can lead to mortality with or without associated tissue lesions and gill injury (Black et al., 1991). Gill lesions, including excessive mucous, epithelial necrosis, hyperplasia, oedema, haemorrhage, and congestion have been reported after exposure to several different algal species (Kent et al., 1995; Mitchell & Rodger, 2007; Rodger, Henry, et al., 2011; Treasurer et al., 2003; Yang & Albright, 1992). Gill lesions have been suggested to be caused by direct physical damage (mechanical injury/irritation), toxicity and supersaturation of seawater caused by algal photosynthesis (gas-bubble trauma) (Rodger, Henry, et al., 2011). In addition to mortality related to gill damage, HAB mortalities can occur due to production of a variety algal toxins affecting other organ systems, oxygen depletion, physical clogging of gills and increased water viscosity (Burkholder, 1998). Most reports concerning impact of harmful algal blooms on farmed Atlantic salmon describe acute mortality and disease. Relatively little is known about potential subclinical and chronic effects of algal blooms on gill health, although it has been shown that exposure to sublethal levels of algae may impact outcomes of concurrent infectious disease (Albright et al., 1993).

Jellyfish and other cnidaria (gelatinous zooplankton)

Jellyfish and other cnidaria can have a negative impact on fish health and lead to mortality and losses. All cnidarians have explosive cells (cnidocytes) containing stinging nematocysts (cnidocysts) with toxins of variable chemical composition and potency (Helmholz et al., 2010). Several different classes have been implicated in cases of gill damage and/or fish mortality. Amongst these are hydromedusae (e.g., *Solmaris corona, Phialella quadrata*), siphonophores (e.g., *Muggiaea atlantica*), scyphozoans (e.g., *Cyanea capillata, Aurelia aurita, Pelagia noctiluca*) and hydroids (e.g., *Ectopleura larynx*) (Clinton et al., 2021; Rodger, Henry, et al., 2011). In Norway lion's mane jellyfish (*Cyanea capillata)* and moon jellyfish (*Aurelia aurita)* are common along the entire coastline and local blooms are observed yearly (Figure 11) (Hosia et al., 2014). The stinging bluefire jellyfish (*Cyanea lamarckii)* can be found in the southern parts Norway. Compass jellyfish (*Chrysaora hysocella),* barrel jellyfish *(Rhizostoma octopus)* and helmet jellyfish (*Periphylla periphylla),* and a wide range of other cnidarians are also regularly observed (Eriksen et al., 2012; Halsband et al., 2018; Knutsen et al., 2018; Yaragina et al., 2021).

Cnidarians can cause problems for fish farms in several ways. Stinging nematocysts can directly damage fish surfaces. The resulting disruption of skin and gill barriers can allow for secondary infections and jellyfish may serve as vectors for bacteria. Lastly, high numbers of medusa can lead to depletion of water oxygen and suffocation of fish (Clinton et al., 2020; Delannoy et al., 2011; Ferguson et al., 2010; Rodger, Henry, et al., 2011). Gill injuries have been described in exposure experiments for jellyfish species *Cyanea capillata* and *Aurelia aurita* (Baxter, Sturt, et al., 2011; Powell et al., 2018). Gill lesions associated with cnidarian exposure include thrombi, haemorrhage, necrosis, epithelial hyperplasia, inflammation, and lamellar oedema (Baxter, Rodger, et al., 2011; Baxter, Sturt, et al., 2011; Baxter et al., 2012; Marcos-Lopez et al., 2016; Mitchell et al., 2011; Powell et al., 2018). Secondary bacterial infections have also been reported in field cases (Ferguson et al., 2010; Marcos-Lopez et al., 2016). As for HABs most reported incidents of fish disease and mortality associated with cnidarian exposure are acute events. Potential chronic, subclinical, and mild to moderate clinical effects of gelatinous zooplankton remains poorly documented in Norwegian salmonid mariculture.
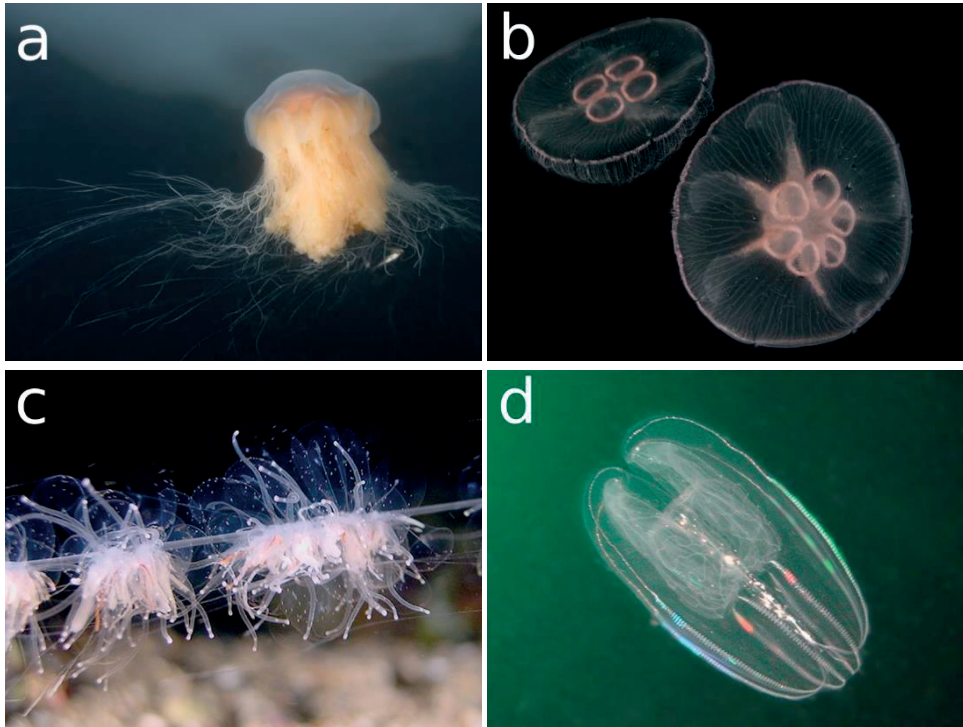
Figure 11. Selected cnidarians and a comb jelly found in Norwegian waters. a) Lion's mane jellyfish and b) moon jellyfish are the most common species of large jellyfish. c) The siphonophore Apolemia uvaria is not native to Norwegian waters, but sporadically appear and have caused large losses of farmed fish. d) Bolinopsis infundibulum belongs to the phylum Ctenophora (comb jellies) which do not possess stinging cells and are no longer classified as cnidarians. All images copyright and used with permission from Kåre Telnes (www.seawater.no).

Miscellaneous non-infectious gill disease

A wide range non-infectious factors including medicinal compounds, pesticides, disinfectants, detergents, metals, gases, pollutants, organic and non-organic particles have been suggested or shown to impact gill and fish health as reviewed by Kjelland et al. (2015); Mallatt (1985); Rodger, Henry, et al. (2011); Schumann & Brinker (2020). Nutritional deficiencies can lead to gill pathology (Rodger, Henry, et al., 2011). Deformities of opercula and gill filaments can occur because of aberrant development related to environmental, managerial, nutritional, or genetic factors, but also due to injuries sustained at any life stage (Rodger, Henry, et al., 2011; Sadler

et al., 2001). Neoplasms can develop in the gills of farmed salmon, but are exceedingly rare (Martineau & Ferguson, 2006).

5.1.5.3 Management factors and gill health

Biofouling and *in situ* net cleaning

In aquaculture, biofouling is unwanted accumulation of living organisms on submerged artificial surfaces (Bloecher, 2013). Biofouling can have a range of negative effects, both on the integrity of the net pens, water exchange and oxygen levels. Biofouling may affect cleaner fish efficacy and serve as a reservoir of fish pathogens (Bloecher & Floerl, 2020; Imsland et al., 2015). Biocide coated nets in combination with regular *in situ* net cleaning are commonly used to control biofouling growth on net pens in Norwegian aquaculture. In parts of the country with high levels of biofouling an average of 15 cleaning events are required per production cycle (Bloecher & Floerl, 2020).

In situ net cleaning generates particles consisting primarily of biofouling organisms and to a lesser extent antifouling coating (Bloecher et al., 2019; Carl et al., 2011; Floerl et al., 2016). The cnidarian *Ectopleura larynx* (syn. *Tubularia larynx*) is one of the species dominating the biofouling communities on net pens in Norwegian waters. The highest load of this organism is found between August and November (Bloecher, 2013; Bloecher et al., 2015; Bloecher et al., 2019; Bloecher et al., 2013; Guenther et al., 2009; Guenther et al., 2010; Napsøy, 2020). Fish experimentally exposed to biofouling debris consisting of *E. larynx* fragments had a higher prevalence and higher average number of lamellar thrombi in the gills after exposure (Bloecher et al., 2018). In a second laboratory study, focal areas of epithelial sloughing, necrosis, and haemorrhage was found in both exposed and control groups (Baxter et al., 2012). In contrast, no effect on gill health was found in a field trial sampling fish from commercial sea farms before and after *in situ* net cleaning (Napsøy, 2020). However, no other studies have examined the effect of *in situ* net cleaning on gill health in commercial sea farms.

Non-medicinal delousing

Sea lice infections in Norwegian salmonid farming were initially controlled by medicinal compounds but widespread resistance has led to a shift to non-medicinal delousing methods (NMMs), i.e., thermal, and mechanical treatments (Myhre Jensen et al., 2020; Overton et al., 2018; Sviland Walde et al., 2021). Two systems for thermal delousing and three different systems for mechanical delousing are currently commercially available in Norway. In addition, freshwater bath treatments alone or in combination with other NMMs have been used with increasing frequency for the last few years (Jensen et al., 2022; Sommerset et al., 2021). Thermal delousing involves submerging fish in water with a temperature of 28-34 ℃ for 20-30 seconds (Holan et al., 2017; Noble et al., 2018). Mechanical delousing involves flushing fish with water jets, flushing and brushing, or negative pressure and turbulence combined with flushing. Medicinal delousing with hydrogen peroxide baths is still used to a lesser extent. Hydrogen peroxide can lead to gill injuries and mortalities dependent on substance concentration, treatment duration and ambient water temperature (Kiemer & Black, 1997; Speare et al., 1999). All treatments occurring outside the net pen, both NMMs, medicinal and non-medicinal bath treatments, involve crowding and pumping that may lead to stress, risk of hypoxia and mechanical injuries to the fish (Erikson et al., 2018; Gismervik et al., 2017; Gismervik et al., 2016; Grøntvedt et al., 2015; Oppedal et al., 2011; Roth, 2016; Skjervold et al., 2001).

Initial field studies indicated that fish welfare and treatment effect of thermal delousing was acceptable, while effects on welfare were somewhat variable for mechanical delousing (Erikson et al., 2018; Gismervik et al., 2017; Grøntvedt & Kristensen, 2018; Grøntvedt et al., 2015; Nilsen et al., 2010; Roth, 2016). However, increased mortality and injuries or lesions have been observed in gills, brain, eyes, thymus, vomeronasal bone, heart, skin, and fins after thermal and/or mechanical delousing (Hjeltnes et al., 2018; Poppe et al., 2018; Poppe et al., 2021; Sviland Walde et al., 2021). Gill lesions described include aneurysms, haemorrhages, and thrombi as well as non-specified mechanical injuries (Gismervik et al., 2019; Gismervik et al., 2017; Jørgensen & Rød, 2019; Poppe et al., 2018; Sommerset et al., 2021). Results from field and laboratory trials have yielded conflicting results about whether thermal and mechanical delousing cause gill injury (Ellingsen & Moljord, 2019; Gismervik et al., 2019; Gismervik et al., 2017; Jørgensen & Rød, 2019; Kvåle, 2020; Mangor-Jensen et al., 2017; Moltumyr et al., 2021; Moltumyr et al., 2022). Few field studies have included quantitative, or semi-quantitative microscopic assessment of

gill tissue, explored potential differential gill gene expression, or considered potential cumulative, indirect, or long-term effects of non-medicinal delousing on gill health.

5.1.5.4   Understanding the causes of gill disease

A wide range of infectious agents, managerial and environmental factors have been shown or suggested to cause gill pathology and gill disease, as shown by the review in the previous segments. However, it is important understand that an association or a correlation between an infectious agent and a disease is not sufficient evidence to conclude that the agent is the cause of the disease. It should also be noted that whether a factor can cause gill disease, and whether it is an important cause of gill disease or pathology in sea farmed salmon, are two separate questions. To clarify and expand on this, causal associations are discussed in the following segment.

5.1.6   Establishing causality

How causality is established will vary depending on the type of disease and its cause(s). Koch's postulates (Table 1.) were proposed as criteria to establish that an infectious agent cause a specific disease (Koch, 1882; Koch, 1982, as cited by Méthot & Alizon, 2014). Koch's postulates rely on experimental evidence to link an organism to a disease. Fulfilling the original postulates requires that the infectious agent can be successfully isolated, cultured and transmitted to a new host. The postulates have been fulfilled for a wide range of microorganisms and diseases, but it was recognized early on that the criteria were too rigid (Rivers, 1937). Modified Koch's postulates have been suggested to adapt to new microorganisms and molecular technologies, to account for variation of pathogenicity within different strains of the same species or to consider contribution of the host's immune status to disease development (Falkow, 1988; Fredricks & Relman, 1996; Greenberg et al., 2006; Rivers, 1937). Experimental evidence using randomized control trials to determine if an exposure (such as an infectious agent) has an effect is considered the gold standard to identify causal associations (Dohoo et al., 2014a). In such experiments the exposure will always

precede any possible outcome, and other confounding variables affecting the outcome are made independent by randomization.

Observational studies are alternative methods to determine if an association between an exposure and an outcome exists. Observational studies occur in "real world" settings and the relationship between multiple exposures can be evaluated. This is advantageous when considering that both host and environmental factors, as well as co-infections, can determine whether a specific infectious agent is associated with disease. Hill (1965) proposed a set of nine criteria to separate causal from non-causal associations in observational studies. These can be applied to both non-infectious and infectious factors (Table 1.). Some of the original criteria may not be very helpful. Lowe et al. (2008) listed only six principal criteria, excluding the criteria of analogy, experimental evidence, and coherence, when using Hills criteria to formulate questions relevant to the study of Crohn's disease. As described by Rothman and Greenland (2005) and Dohoo (2014a) Hill's criteria have limitations and their usefulness and how to apply them is debated among researchers. However, these criteria (Table 1) can serve as a useful starting point when discussing causality and the four gill pathogens *N. perurans*, *Desmozoon lepeophtherii,* salmon gill poxvirus and *Candidatus* Branchiomonas cysticola in sea farmed Atlantic salmon.

Classic Koch's postulates	Hill's criteria of causality	Questions for gill pathogens
1. The specific causative agent must be found in every case of the disease	Strength of association	Is there a strong risk of disease after infection? Is there a strong association between infection and gill disease or pathology?
2. The disease organism must be isolated from a diseased animal and grown in pure culture	Consistency	Has an association between the agent and gill disease been found in different populations under different circumstances?
3. Inoculation of a sample of the culture into a healthy, susceptible animal must produce the same disease (same lesions and clinical signs)	Specificity	Is the agent associated with only one clinical syndrome? Is the agent associated with only one or a limited number of characteristic gill lesions?
4. The organism must be re-isolated from the experimentally infected animal	Temporality	Does infection precede gill disease or do increases in pathogen load precede pathology?
	Biologic gradient Dose-response	Is there a dose-response effect in which a high pathogen load is associated with more severe gill pathology or clinical signs of disease?
	Biological plausibility[1]	Does it make sense that the agent can cause gill disease in the context of current biological knowledge?
	Coherence[1]	Is a cause-and-effect interpretation for an association between gill disease and the agent not in conflict with what is known of the natural history and biology of the disease?
	Experimental evidence	Have experimental studies shown gill disease development following infection?
	Analogy[1]	Is there supporting evidence that a similar microorganism cause gill disease in fish?

Table 1. Koch's postulates, Hill's criteria of causality and gill disease. *Questions adapted to infectious agents involved in gill disease. [1] indicates criteria that are considered less relevant and based on prior beliefs.*

5.1.7 **Knowledge gaps**

As the review above shows, progress has been made in identifying and characterizing potential aetiologic agents of gill disease, and in exploring the impact of different agents, environmental and managerial factors on gill health. However, several knowledge gaps remain, and the changing managerial practices may have introduced new health challenges. While *N. perurans* is established as the causative agent of amoebic gill disease, the importance of microorganisms *D. lepeophtherii*, *Ca.* B. cysticola and salmon gill poxvirus in development of gill disease in Norwegian sea farmed Atlantic salmon is still unclear. Information about the temporal development of gill infections in Norwegian sea farmed salmon and how the different pathogens, environmental and managerial exposures might interact to affect gill health is also largely missing.

5.1.8 **Aims and objectives**

The overall aim of the thesis was to investigate the impact and interactions of selected microorganisms, environmental and managerial factors on gill health of farmed Atlantic salmon. To achieve the overall goal and address the identified knowledge gaps, the following sub-goals were formulated:

1. To describe the temporal development of prevalence and load of *N. perurans*, *Ca.* B. cysticola, *D. lepeophtherii* and SGPV in cohorts of sea farmed Atlantic salmon (Paper I).
2. To determine if variation in pathogen prevalence and load of *N. perurans*, *Ca.* B. cysticola, *D. lepeophtherii* and SGPV had an impact on gill health, as measured by gill-related mortality, gross gill scores and extent of tissue damage detected by histopathology (Paper I).
3. To determine if the water levels of phytoplankton and gelatinous zooplankton had an impact on gill health, as measured by gill-related mortality, gross gill scores and extent of tissue damage detected by histopathology (Paper I).
4. To examine if *in situ* net cleaning as performed in commercial aquaculture can have impact on gill health of farmed Atlantic salmon as measured by extent and prevalence of microscopic gill lesions (Paper II).

5. To explore if thermal and mechanical delousing performed in a commercial setting can lead to acute gill damage, differential gene expression, and changes in pathogen prevalence and load in the gill tissue of farmed Atlantic salmon (Paper III).

5.2 Summary of Papers

5.2.1 Paper I: A cohort study of gill infections, gill pathology and gill-related mortality in sea farmed Atlantic salmon (*Salmo salar* L.): Descriptive analysis

We performed a prospective cohort study, following groups of Atlantic salmon in Western Norway with repeated sampling and data collection from the hatchery phase and throughout the first year at sea. The objective was to determine if variation in pathogen prevalence and load, and zoo- and phytoplankton levels had an impact on gill health as measured by gill-related mortality, gross gill scores and extent of tissue damage detected by histopathology. Further, to describe the temporal development of pathogen prevalence and load, and gill pathology, and how these relate to each other. In the freshwater phase fish from 8 fish-groups were sampled 0 to 3 times for histopathology (n=350) and PCR-analysis for SGPV and *Ca.* B. cysticola (n=378). These 8 fish-groups were split into 2 pens after sea transfer. Thus, 16 fish-groups originating from 4 hatcheries (2 RAS, 2 FT) were transferred to 8 sea farms in autumn 2018 and spring 2019. Each fish-group were sampled 6 to 10 times for histopathology (n=3897) and for PCR-analysis for SGPV, Ca. B. cysticola, *D. lepeophtherii* and *Neoparamoeba perurans* (n=3933). Gross gill scoring of 20 fish per fish-group were planned to be performed weekly during the sea phase, but between 6 to 50 fish were scored per time point and fish-group, with scores available from 33 to 51 weeks across all fish-groups (n=15553). Production data were collected for the entirety of the sea phase, with total mortality and cause-specific mortality recorded daily. Water samples from each sea site (n=29-56 per site) were examined for presence of gelatinous zooplankton (n=323) and phytoplankton (n=322). A descriptive analysis was made based on graphical presentations of the data, assessing temporal development and covariation of gill outcomes and the exposures on group-level. Association between pathogen load and observation of pathogens in tissue sections, and between pathogen load and gill pathology were examined by producing scatter plots and box plots, as well as calculation of Spearman's Rank-Order Correlation coefficient or by use of logistic regression models.

Neoparamoeba perurans appeared to be the most important cause of gill disease in the study population. High loads of the pathogen coincided with a high prevalence of amoebic gill disease diagnosed by histology, moderate to severe gill pathology and a

period of gill-related mortality. There was a correlation between the levels of epithelial hyperplasia and the pathogen load (Ct-levels) of *N. perurans* detected in the gill tissue. There was strong a correlation between the proportion of fish with amoeba and AGD diagnosed on histology and the prevalence and load of *N. perurans* detected by PCR. No clear association and low or no correlation was found between extent of hyperplasia and total extent of gill pathology and agent load for putative gill pathogens *D. lepeophtherii*, SGPV and *Ca.* B. cysticola. There was low or no correlation between pathogen load for any pathogen and extent of gill vascular lesions or overlap of hyperplastic and vascular lesions. There was a positive association between load of SGPV, *N. perurans* and *Ca.* B. cysticola and the number of fish with epithelial cell necrosis/apoptosis observed on histology. The probability (odds) of observing epithelial necrosis in the gills of fish with a moderate load of SGPV was 21.60 times higher compared to fish testing negative for SGPV. Histopathology seemingly unrelated to any of the four infectious agents was found in some fish-groups. Infection with *Pasteurella atlantica* genomvar *salmonicida* caused pasteurellosis, gill pathology and mortality in one fish-group. There was no covariation between the levels of gelatinous zooplankton and phytoplankton detected in sea water samples and any of the gill indicators. High levels indicative of blooms or swarms of harmful species were not detected or reported in the study period.

Co-infection with three more putative gill pathogens was common across all sites. All fish-groups were infected with all four infectious agents at some point, even if most groups did not develop severe gill pathology or gill-related mortality. There was moderate correlation between pathogen load (Ct-levels) of *N. perurans* and *D. lepeophtherii,* but weak or low correlation between the other pathogens. All fish-groups were infected with *D. lepeophtherii* and *Ca.* B. cysticola after sea transfer. No clear seasonal variation in pathogen load or prevalence was observed after infection. There was a seasonal variation in pathogen prevalence and load of *N. perurans*. The amoeba was detected in late summer, autumn, and winter, while samples were negative in spring and early summer. SGPV infection was detected after sea transfer in all fish-groups, independent of infection status at sea transfer. There was a seasonal variation in prevalence with most SGPV infections detected during late summer and fall. These observations suggest that SGPV infection spreads horizontally at sea and could indicate that previous infections with SGPV does not protect against reinfection. Fish-groups at the same sea sites were more similar in terms of prevalence and severity of gill infections, gill pathology and gill-related mortality than fish at other sea sites. There was an overall weak correlation between gross gill scores and extent

of histopathology in the study, and there was no increase in gross gill scores in the time period when the most severe gill pathology and gill-related mortality was observed.

5.2.2 Paper II: Assessment of acute effects of i*n situ* net cleaning on gill health of farmed Atlantic salmon *(Salmo salar L)*

Laboratory trials have shown that fragments of the common net fouling organism *E. larynx* can cause gill lesions in Atlantic salmon and net management practices have been considered a possible factor affecting gill health in the sea phase of salmon farming. We performed a field trial with repeated sampling of 30 fish from 3 pens before and after net cleaning (n=270) to determine if exposure to net cleaning debris generated during a real-life *in situ* net cleaning event could lead to gill lesions. Two pens were moderately fouled, and one had a low degree of fouling. Overall, very few lesions and a small extent of gill tissue with pathology was found both before and after *in situ* net cleaning. No impact of net cleaning on mortality rate, specific feed rate or specific daily growth rate was observed. When comparing all fish and pens sampled after cleaning with fish sampled before cleaning there was no significant difference in the number of fish with epithelial hyperplasia or vascular lesions, nor any of the other recorded categories of lesions. The probability (odds) of fish from moderately fouled net pens having subacute vascular damage (thrombi) in the gills at one day after net cleaning was 2.36 (95% CI 1.21-5.71) times higher than fish sampled before net cleaning. No significant difference was found between fish sampled before and eight days after cleaning of the moderately fouled pens.

5.2.3 Paper III: Effects of thermal and mechanical delousing on gill health of farmed Atlantic salmon (*Salmo salar* L.)

In this study the aim was to determine if thermal and mechanical delousing in a commercial setting can lead to gill damage, differential gene expression, and changes in pathogen prevalence and load in the gill tissue of farmed Atlantic salmon. Gill tissue from 29 to 30 presumed healthy fish from three pens at one farm site was collected prior to and at two time points post-thermal (n=269) or mechanical delousing

(n=270). All gill samples were examined by microscopy. Gill tissue from fish undergone thermal delousing were analysed by RT-qPCR-analysis to determine levels of mRNA expression for markers of heat shock responses, hypoxia, inflammation, and repair (n=142). RT-qPCR-analysis to determine prevalence and load of gill pathogens *Candidatus* Branchiomonas cysticola and *Desmozoon lepeophtherii* (n = 198), and *Neoparamoeba perurans* and salmon gill poxvirus (n=110) was also conducted. There was an increased number of lamella and percentage of gill tissue with vascular and hyperplastic lesions observed post-treatment for both delousing methods, though the overall percentage of gill tissue with pathology generally was low (<2%). The distribution, dominating type of vascular lesion, and the presumed temporal development of lesions was different for the two treatments. An increased load of *Ca.* B. cysticola and differential expression of genes involved in pathways of cell stress, inflammation, repair, and proliferation was detected in the gill tissue after thermal delousing. There was an increase in the number of fish with microorganisms and lesions possibly associated with pathogens observed in the gill tissue after treatments. The percent daily mortality and mortality rates increased during and after delousing for all pens, and most fish died within days after treatment.

5.3 Material and methodological considerations

5.3.1 Ethical considerations

All the studies reported here are field trials performed at commercial aquaculture facilities in Norway. All treatments and operations fish were submitted to were performed as part of regular management operations at the sites. Sampling, handling, anaesthesia, and euthanasia of the fish used was performed according to the farming companies standard operating procedures and national guidelines (Akvakulturdriftsforskriften, 2008, § 34. Avlivning av fisk). Because no manipulation of the fish was done for the purpose of research and fish were euthanized using approved methods, we were not obliged to apply for permission for use of animals in research for the studies described in this thesis.

5.3.2 Study populations

Study populations and materials examined as a part of this thesis and in the papers originate from different sites for each paper (Table 2). For examination of effects of *in situ* net cleaning, thermal and mechanical delousing (Papers II and III) three fish-groups (pens) were repeatedly sampled at three different sea sites, one site for each exposure.

For the cohort study (Paper I), fish and fish-groups originated from 4 different freshwater facilities with 2 cohorts from each facility (8 fish-groups). Freshwater sites were selected to include sites using flow-through systems (FT) and recirculating aquaculture systems (RAS). Two sites with a history of gill disease (sites 3 and 4) and 2 sites without known gill issues (sites 1 and 2) were included. The fish-groups were sea transferred autumn 2018 (S0, 4 fish-groups) and spring 2019 (S1, 4 fish-groups). Each fish-group were split into 2 separate pens at transfer leaving 16 fish-groups during the sea phase (Figure 12). Sea sites were selected to include 4 sites with a history of problems with gill disease and gill-related mortality (sites A, C, D and G). The remaining 4 sites only had mild or no recorded historic gill-related mortality.

Paper	#Sites/sea farms	#Fish-groups	Time of study	Exposure	Material, methods, and data
I – Cohort study	8 A-H	16 fish-groups/2 per site	>2 years	Pathogen levels Delousing operations Plankton levels Sea temperature Season	Gross gill scoring Histopathology Rt-qPCR for gill pathogens Water analysis for plankton Cause-specific mortality Total mortality Sea temperature Fresh water data Delousing events
II – In situ net cleaning	1	3 fish-groups (pens) A-C	~10 days	*In situ* net cleaning	Histopathology Total mortality Net scoring Information on net cleaning
III – Thermal and mechanical delousing	2	3 fish-groups (pens) per site and treatment A-C and D-F	~10 days for each treatment	Thermal and mechanical delousing	Histopathology Total mortality Rt-qPCR for gill pathogens* Gene expression analysis* Information on delousing methods

Table 2. Overview of sites, fish-groups and material, methods, and data. *Sites and fish-groups used in studies I-III does not overlap. *Analysis only performed for fish treated with thermal delousing.*
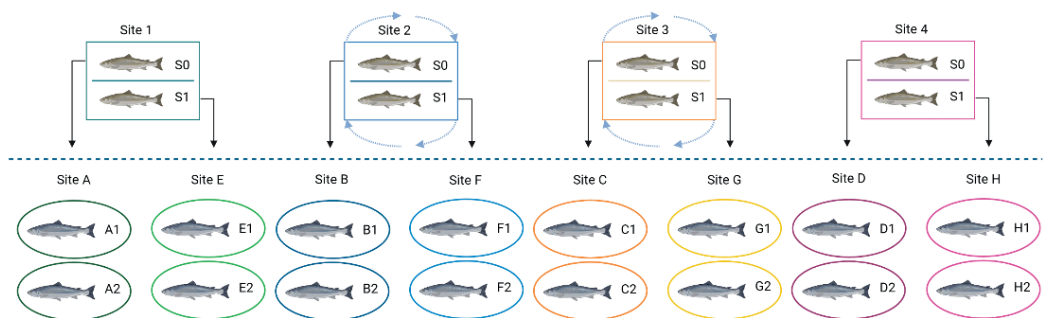
Figure 12. Overview of fish-groups in the cohort study. *The study included freshwater sites (n=4), sea sites (n=8) and fish-groups (n=16). Stocking period (S0=autumn stock/S1=spring stock) and water treatment (flow-through vs. RAS=circular arrow) is indicated (Figure created in BioRender.com).*

The main study unit in the cohort study was the 'fish-group', defined as fish from the same hatchery transferred to sea at the same time and to the same cage. However, parallel gross scores, histopathology, and RT-qPCR-results from individual fish additionally allowed for examination of correlation between tissue lesions, pathogen prevalence and load using 'fish' as the study unit. Similarly, the individual fish was the study unit in the statistical analysis described in paper II and III, even if all fish were representatives of the respective fish-groups (pens) they were sampled from. For these two papers a set of selection criteria was used to include fish-groups that had not been exposed to the respective interventions previously and were presumed healthy at the outset of the study.

Unfortunately, mixing of four fish-groups with other fish-groups at the sea sites occurred during the cohort study. These mixing events may have impacted all results of the scoring and sampling points following mixing, and ideally farms would have dropped mixing of study groups with other fish-groups. Luckily, fish at the same farm appeared to be more similar in terms of infections and pathology compared to fish at different farms. Further, after most mixing events the original fish-groups made up a large proportion of the new fish-group. For these reasons, we chose to report the results, and inform the readers about this weakness of the study.

5.3.3   Study design

A prospective cohort study with repeated sampling was performed to describe the development of gill infections and associations between environmental factors, gill pathogens, gill-related mortality, and gill pathology (Paper I). The aim was to detect the potential impact of several exposures on gill outcomes. For some of these exposures the exposure status was permanent, known in advance, and fish-groups were selected based on this (e.g., whether fish were kept in RAS or flow-through systems and timing of sea transfer). Other exposures were non-permanent, and it was unknown if the fish-groups would experience these during the study period (e.g., high plankton levels or net cleaning events). Major advantages of cohort studies are the possibility to evaluate the relationship between multiple exposures and the development of disease, and to observe change over time. The sequence of events can be established, and it may be possible to identify and link events (like gill disease) to

a particular exposure (Caruana et al., 2015). A major disadvantage of prospective cohort studies is that it is not possible to know in advance if the outcomes of interest will occur during the study period. In addition, the cost and effort involved are high (Caswell et al., 2018a). In this study most fish-groups did not develop severe gill pathology or gill-related mortality, which limits our possibility to draw conclusions about possible factors associated with gill disease. Inclusion of more fish-groups might have prevented this issue but would have added significant cost and time.

The studies performed to determine if *in situ* net cleaning, thermal and mechanical delousing (Paper II and III) influenced gill health were historical control trials. In this study design the outcomes after an intervention are compared with the levels of the outcomes before the intervention. Our studies involved repeated sampling of gill tissue for histopathology assessment, RT-qPCR-analysis, and gene expression of three pens before and at given time points after delousing and net cleaning. This study design can be problematic as certain criteria must be fulfilled for study results to be valid (Dohoo et al., 2014b). The criteria were largely met in our studies as we had chosen which outcomes to measure in advance, collected samples and data before and after the interventions and used the same methods and criteria to measure the outcomes in all samples. Major environmental changes or insults were not recorded during the study period, and the studies were performed over a short time period. However, an impact of environmental and infectious factors on gene expression and histopathology outcomes cannot be ruled out. Because the aim of these studies was to assess the effects of i*n situ* net cleaning, thermal and mechanical delousing as it is performed in a commercial setting, performing a controlled laboratory trial was not an option. A randomized controlled field trial including untreated control groups from the same sea sites would have been the "gold standard" for proving causal association, because confounding effects could be minimized. However, this would require leaving the control groups untreated or subjecting them to a sham treatment, crowding the fish and moving them through the treatment unit without exposure to heated water or water jets. This option was considered ethically and financially unfeasible. It would also be a practical issue because farm sites often delouse and clean pens of all fish-groups at a site simultaneously.

All the studies described here are field trials in commercial sites and managerial events performed as a part of regular salmon production. This means that our results are likely to be highly valid for the fish farming industry. However, the geographical distribution of the included farms (Western Norway), means that the results of the

cohort study might be less valid for fish farms in northern parts of Norway. In addition, the managerial practices, environmental factors, and infectious agents involved in development gill disease may vary markedly in the different parts of the world where salmonid mariculture occurs, and this should be considered when evaluating the generalizability of results of studies for a given location or country.

5.3.4   Sampling

Sampling during the cohort study (Paper I) was planned to be performed at three time points evenly spaced throughout the freshwater phase and at approximately the same time for each fish-group. Similarly, the aim was to sample the same number of fish and collect water samples and perform gross scores at evenly spaced intervals during the sea phase of production. Unfortunately, it was not practically possible to get samples from the entire freshwater phase, nor samples from all fish-groups. During the early part of the project and after sea transfer of the S0 fish-groups changes were made to the sampling protocol and an additional fish-group was added for each site. Later, poor weather, practical issues at the farm sites and the corona pandemic complicated sampling and lead to delays. Thus, the number of samples available, the number of sample points and the sampling intervals varies across and within fish-groups. The time-interval between each sampling point after sea transfer (20 to 122 days) also means that several changes can occur in terms of treatments, environmental exposures and pathogen load and prevalence between each sampling point. Water samples for plankton analysis were collected more frequently, sometimes as frequently as one week apart during the periods with the highest risk of blooms. However, plankton levels can fluctuate markedly even during a 24 hour period, and with our sampling regime blooms and swarms may have been missed.

All management operations (net cleaning and delousing) were recorded by the farming company for fish-groups included in the cohort study. However, the likelihood of detecting potential negative effects of such treatments will be impacted by the timespan between the treatment and the subsequent sampling point. Additionally, if the pre-treatment samples were collected a long time prior to the treatment, other infectious and non-infectious factors could have influenced the gills even before delousing occurred. Thus, to detect potential negative effects of non-medicinal delousing and *in situ* net cleaning on gill health three additional field trials

were performed. For each trial gills were sampled 1 to 2 days prior to exposure, and 1 to 2 days and 6 to 9 days after exposure. Sampling of all fish-groups at the exact same time point in relation to the treatment/net cleaning would have been ideal. This was not practically possible due to resources and staff necessary to perform treatments and the variable timing of treatments.

The aim for all studies (Paper I-III) was to sample fish that were representative for the status of the fish-group. To achieve this the ideal would have been random sampling where each fish in a pen is equally likely to be selected for sampling (de Blas et al., 2020). However, simple random sampling or systematic sampling is not practically possible when trying to catch fish from a 30 meter deep pen containing more than 150 000 individuals (Nilsson & Folkedal, 2019; Stephen & Ribble, 1995). Thus, sampling for any of the studies reported here were not truly random.

For each paper it was decided to sample up to 15 fish with and 15 without clinical signs of disease to have a better chance of detecting gill disease or gill injuries if it occurred in the fish-groups. However, for a large proportion of sampled fish in the cohort study the health status was unknown, and very few were recorded as moribund or sick. Prior to sampling at sea, fish were crowded using feeding and a purse seine. In reality, the 30 first fish taken up from the seine with a dip net were sampled. It is possible that fish with better health than the general population were sampled as diseased fish may become anorexic and will not be attracted to food. On the contrary, weak, sick, and slow fish at the surface may have an increased risk of being trapped by the seine and caught with the dip net (Stephen & Ribble, 1995).

From each fish the second left gill arch was sampled for histopathology and third left gill arch was sampled for RT-qPCR. Such a standardized sampling is necessary to compare results across groups and time points. In contrast, if the primary aim had been to determine the prevalence of gill disease and gill infections in the fish-groups, sampling of the grossly diseased gill arch and tissue within randomly selected fish would be the best approach. Thus, the sampling regime may have led to an inaccurate estimate of the prevalence and severity of gill disease and pathology in our studies. This is due to the possibility of sampling fish different from the general population in the pen, and due to the standardized sampling of tissue within each fish (Nilsson & Folkedal, 2019; Stephen & Ribble, 1995). The sampling of different gill arches for RT-qPCR and histopathology may also explain some of the discrepancy between observation of amoeba in tissue sections and detection of *N. perurans* by RT-qPCR.

5.3.5   **Production data**

5.3.5.1   **Mortality data**

Mortality data from all sites were provided by the farming companies (Paper I-III). This included the total number of dead fish per day (total mortality) and the number of stocked fish per day for each site and pen for all studies. With these data it was possible to examine the impact of *in situ* net cleaning and delousing treatments on the overall daily mortality (Paper II and III). Cause-specific mortality was only provided for the cohort-study (Paper I).

Cause-specific mortality was recorded by site staff daily assigning likely causes of death based on gross examination of dead fish and knowledge of infectious disease and management events occurring at the site. Information from laboratory reports and advise from fish health personnel may also have been used when categorizing mortalities. Dead fish were categorized as gill-related mortalities if any type of gill disease or injury were believed to be the cause of death. Because only one cause of mortality could be assigned to each fish, it is possible that fish with gill disease and gross gill pathology were assigned to other mortality categories. It is common to assign all mortalities occurring during and for a short time after treatments as treatment-related, even if infectious disease likely contributed to the fish dying in many cases. Diseases and insults that do not lead to grossly apparent or specific lesions are also likely to be underestimated as causes of mortality. Because of these limitations the cause-specific mortality data will have limited usefulness for studies of the interactions between co-infections, or interactions between infectious, managerial, and environmental factors causing gill disease. The gill-related mortality should be considered a conservative estimate of (severe) gill disease in a population. However, cause-specific mortality classification is inexpensive and can be performed on-site by site staff considering data from concurrent laboratory analysis. Guidelines to ensure uniform registration and coding of different causes of mortality has been published recently (Aunsmo et al., 2021). Using these guidelines different causes of gill-related mortality can be recorded (Persson et al., 2021). This can allow for collection of much larger datasets than reported in this study with less resources spent and may lead to identification of risk factors of gill disease(s) in the future. Because of this potential it was also interesting to include and report the gill-related

mortality and see if there was covariation between gill-related mortality, gross score, and histology results.

5.3.5.2 Managerial factors

For the cohort study (Paper I), information on the date and number of *in situ* net cleaning events, the date and number of delousing events and treatment method were provided by the farming company. Information on water treatment system and management operations in the freshwater phase was also shared. For Paper II and III detailed information on sites, equipment, duration, and any observations made by staff and other persons involved in delousing and net cleaning operations were recorded and shared. For the *in situ* net cleaning study (Paper II) it would have been ideal to perform a detailed characterization of the biofouling community and collect of water samples during net cleaning to quantify the amount and type of fouling particles present in the water and determine how long fish were exposed to these. Unfortunately, we did not have the resources to do this work. Instead, we performed a semi-quantitative scoring of the fouling level based on images of the nets, used fouling scores provided by cleaning operators and examined a sample of biofouling to confirm the presence of *E. larynx* at the site.

5.3.5.3 Environmental data

For the cohort study (Paper I) daily measurements of water temperature was available from all sites, providing useful information on the seasonal variation and how this related to temporal development of gill infections. However, data on water oxygen levels and salinity were not consistently and regularly measured at the different project sites, making comparison between sites difficult. Information on oxygen levels, sea temperature and salinity were provided for the net cleaning, and delousing field studies (Paper II and III).

5.3.6   Laboratory methods

The methods used in this study include morphologic assessment and molecular genetic techniques. For detailed description of the methods used please refer to the respective papers.

5.3.6.1   Histopathologic evaluation

Histopathology is the diagnosis and study of disease of the tissues and involves examining tissues and cells under the microscope. When examining tissue samples sent for investigation of disease outbreaks or for health screening a description of any tissue abnormalities and the resulting diagnoses is provided by the pathologist. To use histopathology results for statistical analysis they need to be transformed into numbers in some way. In the studies reported here two methods are used for this purpose. The incidence method (binomial scoring) involves recording the presence or absence of a lesion or diagnosis resulting in the number and percent of affected fish per fish-group and time point. Secondly, an assessment method combining morphometry (i.e., measurements and counts of lesions) and ordinal data (i. e. estimates of filament orientation as poor, average, and good plane of section) was used to calculate an estimated percent of tissue with microscopic lesions in each gill sample. In addition, the percent of fish with more than 5% gill tissue with lesions was calculated for each fish-group and sampling point yielding another binomial score.

The purpose of the detailed assessment method was to provide higher resolution and more accurate histopathology data than would have been possible using ordinal scoring systems. This allowed us to detect changes in extent of tissue damage that might have gone undetected using an ordinal scoring system. Crissman and colleagues (2004) proposed that any assessment (scoring) method should meet three criteria: a) It should be definable, b) it should be reproducible, c) it should produce meaningful results. To meet the first criterium we carefully described the method and defined each category of lesions recorded or counted. The description and definitions are also expected to improve the reproducibility and understanding of the work among other researchers (Caswell et al., 2018b; Gibson-Corley et al., 2013). However, the complexity of the method made it time-consuming and sensitive to differences in assessment between pathologists. A test was performed where two different pathologists read the same 90 samples, and one of the pathologists scored the

samples twice. The intraobserver agreement was excellent (Lin's concordance correlation coefficient (CCC) ≥0.78, Pearson's correlation coefficient (r) ≥0.90). The interobserver agreement was moderate to good (Lin's CCC ≥0.41, Pearson's r ≥0.47) for most parameters recorded as counts and percent (Østevik et al. unpublished data). It is well-known that pathology data can have problems with repeatability and interobserver agreement, and that including fewer score categories can improve agreement and repeatability (Caswell et al., 2018b; Cross, 1998; Gibson-Corley et al., 2013). The experience and training of the pathologist performing analysis may impact agreement, with lower agreement for junior compared to senior pathologists (Rousselet et al., 2005). It has also been shown that simple scoring systems leads to a loss of information, and it might be difficult to find the balance between getting the most information from tissue samples and securing high repeatability (Bleich et al., 2004; Morris, 1994). Which of these are considered most important likely depends on purpose of the scoring, and this should be considered when developing a new score system.

With the available data sets it was not possible to test whether the assessment method used produced meaningful results in terms of correlation with clinical signs of gill disease or measurements of gill function (validation of tissue pathobiology). The covariation between gill-related mortality and gross gill scores were assessed in Paper I. In this study these outcomes are less accurate and/or different measures of gill health than histopathological assessment, and thus cannot be used validate the usefulness of the current assessment system. Examination of the correlation between an assessment/scoring system and clinical outcomes in commercially farmed fish is challenging because knowledge about the health status of the individual fish sampled frequently is limited or lacking. This is likely to be more feasible in controlled clinical trials where fish can be closely observed, the environment is controlled, fish are free of disease that are not of interest, and clinical outcomes can be accurately recorded.

To avoid introducing variability caused by differences between pathologists, a single pathologist read all samples from the sea phase of the cohort study (Paper I) as well as the three separate trials on net cleaning and non-medicinal delousing (Paper II and III) (Cross, 1998). Furthermore, samples were also randomized, and pathologists were blinded to sample point and fish-group to reduce bias in interpretation (Paper II and III). The pathologist was also blinded to the results of RT-qPCR-analysis for pathogens, gene expression analysis, gross scoring, and water analysis (Paper I-III). The order in which samples were read were not randomized, samples were not read

over a short time period, nor was the pathologist blinded to the site or fish-group examined in the cohort study (Paper I). Because of the long time period of sampling and large number of samples to be read such steps were not practically possible. This lack of random order and long time period of reading the samples might have led to diagnostic drift and reduced consistency of assessment over time (Caswell et al., 2018b). However, because the design of this study did not rely on comparing case and control samples, or exposed and non-exposed fish, and the timing and number exposures like non-medicinal delousing was unknown to the study pathologist this was considered acceptable.

5.3.6.2 Gross gill scoring and gross evaluation

The term gross gill scoring refers to the macroscopic examination of tissue abnormalities and the scoring system used to quantify the extent of tissue with such abnormalities. Tissue scoring is a method to obtain semiquantitative data from examination of tissues (Gibson-Corley et al., 2013). The principles described for histopathologic assessment and scoring of tissues also applies for gross tissue scoring. An advantage of gross scoring is that more of the gill tissue can be examined than typically submitted for histology, though only the left gill arches was scored in the current study. Ideally all arches would have been evaluated, but this was deemed too time-consuming. The agreement between gross and histological evaluation of gill lesions varies between studies, conditions, and assessment/score methods used (Adams et al., 2004; Boerlage et al., 2022; Bridle et al., 2010; Clark & Nowak, 1999; Krol et al., 2020). Studies and practical experience have shown that mild or diffuse lesions can be difficult to detect grossly (Adams et al., 2004; Clark & Nowak, 1999; Collins et al., 2017). Further, characterizing the type of tissue reaction present in the gills may not be possible using gross examination (Mo et al., 2015). Nonetheless, gross evaluation of tissues is fast, cheap, can be performed on-site and are currently used in the field for monitoring of gill health. Because of this, gross scores were included as a gill indicator in the cohort study. Further, this allowed for assessment of the covariation between the different gill indicators; histology results, gross scores, and gill-related mortality in our fish-groups over time. By recording gross gill scores for each gill arch separately it was possible to examine agreement between gross and histology results for the same arch, as well as for mean gross gill score. Gross scoring was not performed in the remaining studies. It was not expected that net cleaning,

mechanical and thermal delousing would lead to severe lesions easily detectable grossly, so resources were better spent on other types of analysis.

A training session with descriptions of the gross scoring system, and example images, were provided to staff at participating sea farms to try to ensure consistency of gross scoring across sites and make sure the scorers understood the scoring system. However, not all scorers received a training session and the scorers experience in assessing fish health varied within sites and over time. Because most scorers were farm staff, scorers differed between sites, while the two fish-groups at the same site were generally scored by the same staff each time. Thus, the inclusion of multiple gross scorers with variable experience, the variation of scorers over time, across and within sites, and the use of a scoring system that was new to the scorers likely introduced variation into the gross score observations unrelated to actual variation in gross pathology (Adams et al., 2004; Rousselet et al., 2005). This fact, in addition to the histology sampling protocol and the inability to detect mild lesions grossly, may explain the poor correlation and inconsistent covariation between gross and microscopic lesions. However, our results showed that cases of moderate to severe gill pathology and AGD may go undetected if gills are only assessed by gross examination.

5.3.6.3 Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Real-time quantitative PCR (qPCR) is an important method for monitoring, surveillance, and diagnostic investigation of infectious diseases in farmed salmonids. PCR is valuable for assisting with diagnosis of a wide range of diseases in human and veterinary medicine (Bustin et al., 2005). qPCR is an improvement of the original PCR-method whereby simultaneous target amplification and generation of target-specific signal are achieved. The method is highly sensitive, and the amount of target genetic material can be quantified in real-time. Reverse transcription qPCR (RT-qPCR) is used when the starting material is RNA. The first step of RT-qPCR involves reverse transcription of RNA into cDNA. The cDNA is then used as a template for the qPCR reaction. The qPCR-reaction involves repeated cycles of denaturation, annealing and extension leading to amplification of the target DNA/cDNA if it is present in the sample. For each cycle, a fluorescent reporter molecule binds amplified DNA and emits a fluorescence signal proportional to the amount of replicated DNA. The cycle

threshold (Ct) (synonym quantification cycle (Cq)) refers to the cycle number at which enough amplified product is present to yield a detectable fluorescence signal.

All RT-qPCR-analysis for the putative gill pathogens (Papers I-III) was performed at and by Fish Vet Group Norge AS (Oslo, Norway), a Norwegian accreditation approved and certified fish health laboratory. All PCR-analyses were performed on genetic material extracted from sampled gill tissue. The primer and TaqMan probe sequences used originated from publications by other researchers (see Supplementary file 1 Paper III) and each assay was validated in-house before being approved for use in the diagnostic service. Resulting Ct-values for the putative gill pathogens were used to generate ordinal variables and reverse Ct-values representing pathogen load in the cohort study (Paper I) (Downes et al., 2018). Median reverse Ct-values and pathogen prevalence was used to describe the temporal development of infection and relationship between gill pathology and pathogen load. We chose not to normalize Ct-values in this study as reverse Ct-values are easier to understand and relate to for potential readers working with fish health and farming. Further, there is not a natural starting point or day 0 in the cohort study, so comparing PCR-results obtained before and after a specific time point does not make sense.

RT-qPCR using SYBR Green for target amplification of salmonid genes for gene expression analysis (Paper III) was performed at the Norwegian University of Life Sciences (NMBU). In paper III, normalized Ct-values (or more accurately named normalized expression (NE)) and normalized fold change were calculated for pathogens *Ca.* B. cysticola and *D. lepeophtherii* using salmon elongation factor alpha as a reference gene. Relative expression of the selected endogenous salmon genes was calculated using the ΔΔCT method using β-actin as a reference gene (Livak & Schmittgen, 2001). For both pathogens and salmon genes normalized expression of post-treatment samples was compared to the normalized expression of the pre-treatment samples to determine if thermal delousing affected gene expression or pathogen load of the gill tissue.

5.3.6.4   Water analysis for zoo- and phytoplankton

Phytoplankton levels were quantified by examination of the submitted iodine-fixed water sample in a Sedgewick counting chamber slide under an inverted microscope. The number of gelatinous zooplankton was quantified by examination of the

formalin-fixed water sample in a cell culture bottle under a stereomicroscope. Species and genus identification was performed as far as possible.

5.4 Discussion of main results

5.4.1 What is the temporal development of gill infections during the sea phase of production?

Our findings in the cohort study (Paper I) confirmed that *Neoparamoeba perurans* infection and amoebic gill disease in Norwegian farmed salmon follows a seasonal pattern. Observations of the highest pathogen load, prevalence of infection and AGD in autumn and winter, and clearing of infection in spring is consistent with the pattern observed in Norway since AGD first became endemic (Mo et al., 2015; Sommerset et al., 2021). This pattern is likely related to the seasonal variation in sea temperatures which ranged from 4.4 to 16.9°C during the study period (Clark & Nowak, 1999). The finding of lower AGD prevalence at sites with lower salinity is also in line with previous observations (Clark & Nowak, 1999; Mo et al., 2015; Oldham et al., 2016). The autumn-transferred fish-groups followed through two consecutive autumns and winters became infected and developed amoebic gill disease both autumns at sea, though generally mild AGD-lesions were found both years. The prevalence of AGD did not appear to drop markedly the second year at sea in the current study despite earlier reports that fish being repeatedly exposed to the *N. perurans* might develop resistance (Findlay & Munday, 1998; Findlay et al., 1995; Wynne et al., 2008).

In agreement with previous studies from Norway and UK, we found a high prevalence of *D. lepeophtherii* and *Ca.* B. cysticola infection in Southern and Western Norway. Infection was established after sea transfer and persisted throughout the study period amongst all fish-groups (Downes et al., 2018; Gunnarsson et al., 2017; Herrero, Rodger, et al., 2022; Nylund et al., 2011; Steinum et al., 2015; Steinum et al., 2010; Sveen et al., 2012). A near 100% prevalence of *Ca.* B. cysticola and *D. lepeophtherii* was found in the fish-groups undergoing thermal delousing. Intracytoplasmic, intraepithelial bacteria (epitheliocysts) were also found in more than 80% of fish undergoing thermal and mechanical delousing, sampled the second year at sea (Paper III). In contrast, a very low prevalence of epitheliocysts was found among fish from the net cleaning study, sampled shortly after sea transfer (Paper II).

D. lepeophtherii and *Ca.* B. cysticola infection occurred more rapidly after sea transfer for autumn-transferred fish compared to spring-transferred fish. The highest median *D. lepeophtherii* loads of the study period were also seen earlier for autumn-

transferred fish but were observed relatively rapidly during the first autumn at sea for both fish-groups. Conversely, there was no evident seasonal and temperature-dependent variation in pathogen load once fish-groups were infected with these two pathogens. In some fish-groups, the highest loads of *Ca.* B. cysticola were found during the coldest periods of the year. While the highest pathogen loads of *D. lepeophtherii* were found during the first autumn at sea, no consistent increase in load of either agent was seen the subsequent autumn.

A similar difference in timing of *D. lepeophtherii* infection between autumn and spring-transferred salmon and development of agent prevalence and load has been reported previously (Sveen et al., 2012). However, only the spring-transferred fish-group was repeatedly sampled until and including the second summer at sea (Sveen et al., 2012). The highest loads of *Ca.* B. cysticola and *D. lepeophtherii* were observed during summer and autumn in a Norwegian study including six spring-transferred fish-groups/sites with four repeated rounds of PCR-analyses for selected gill pathogens throughout the first year at sea (Gunnarsson et al., 2017). Similarly, a study including two spring-transferred fish-groups at two sites in Scotland identified peak *D. lepeophtherii* loads during the first autumn and winter at sea, while *peak Ca.* B. cysticola loads was found the first summer after sea transfer. However, one of these fish-groups became infected with *D. lepeophtherii* shortly after sea transfer and both fish-groups were infected with *Ca.* B. cysticola in the freshwater phase (Herrero, Rodger, et al., 2022). Sampling was not extended into the subsequent summer and autumn in either of these two studies. In agreement with our findings, Downes et al. (2018) found the highest loads of *D. lepeophtherii* the first autumn at sea in a single fish-group of spring-transferred salmon in a site in the southwest of Ireland. This was followed by a decline during the winter months that continued through the following summer. Peak gill load *Ca.* B. cysticola was found during the winter months, as was the case for some of our fish-groups.

The higher sea temperatures in autumn compared to spring may explain the longer time from sea transfer to infection with *Ca.* B. cysticola and *D. lepeophtherii* for spring-transferred fish in our study. However, the lack of a consistent increase in *D. lepeophtherii* load with increasing temperatures the second summer and autumn at sea seen in several studies indicate that this agent may infect or proliferate at higher levels in naïve fish compared to older and presumably persistently infected fish. The timing of peak *Ca.* B. cysticola loads appear to vary across fish-groups and studies, indicating that the dynamics of *Ca.* B. cysticola infection in sea farmed salmon is not

affected by the seasonal variation in sea temperature once the fish are infected with the bacterium. To which extent infected salmon develop immune responses that control these infections, whether such responses may vary with age and which mechanisms could be involved has not been explored.

In our study SGPV infection at sea appeared to have a seasonal distribution with most infected fish detected in late summer and fall. A seasonal distribution of SGPV has not been reported previously, though only two other longitudinal studies including repeated analysis for SGPV in sea farmed salmon have been published (Downes et al., 2018; Herrero, Rodger, et al., 2022). The detection of SGPV infection after sea transfer in previously negative fish-groups are consistent with previous findings that the virus spreads horizontally and suggests that SGPV transmission also occurs in sea water (Gjessing et al., 2017; Wiik-Nielsen et al., 2017). The disappearance and reappearance of the virus throughout the sea phase is in line with the sporadic positive samples in all fish-groups in Irish and Scottish longitudinal studies (Downes et al., 2018; Herrero, Rodger, et al., 2022). These observations suggest that fish-groups may clear the virus and become re-infected multiple times over a production cycle. This contrasts with the lack of reinfection reported previously and indicates that previous infection does not protect against reinfection of the same fish-group (Gjessing et al., 2018). Another possibility is that the virus persists in the population with a very low prevalence to proliferate and spread when conditions are more favourable.

5.4.2 What is the role of infectious agents *Neoparamoeba perurans*, salmon gill poxvirus, *Candidatus* Branchiomonas cysticola and *Desmozoon lepeophtherii* in causing gill disease in Norwegian sea farmed Atlantic salmon?

The role of infectious agents in causing gill disease was explored in the cohort study (Paper I). In this section our findings and the literature will be combined using Hill's criteria of causality to investigate what is known about the selected agent's causal role in gill disease (Table 3). In addition, the relative importance of these agents in cases of gill disease in sea farmed Atlantic salmon will be discussed.

Neoparamoeba perurans appeared to be the most important cause of gill disease in the study population (Paper I). High loads of the pathogen detected by PCR coincided

with a high prevalence of amoebic gill disease as diagnosed by histology, moderate to severe gill pathology, and a period of gill-related mortality. In the fish-level dataset a moderate correlation between the extent of epithelial hyperplasia and the pathogen load (Ct-levels) of *N. perurans* were detected in the gill tissue. There was a positive association between the number of fish with AGD diagnosed on histology and increasing load of *N. perurans* genetic material in the gills in the fish-group dataset. We found a positive association between *N. perurans* load and number of fish with epithelial cell necrosis, or apoptosis. In contrast, low or no correlation between pathogen load and the extent of vascular lesions or overlap of hyperplastic and vascular lesions was found.

N. perurans is firmly established as the cause of amoebic gill disease (AGD), so it was not surprising that this agent was associated with AGD, hyperplasia and gill-related mortality in our study. Cultures of *N. perurans* have been established and cultured organisms have been shown to induce AGD in naïve, healthy Atlantic salmon, hence fulfilling Koch's postulates (Crosbie et al., 2012). AGD can also be transmitted by cohabitation and by exposure to gill mucus from infected fish. Many challenge trials have been performed since the agent was first cultured (Zilberg et al., 2001; Zilberg & Munday, 2000). There is a strong association between infection and gill pathology, and a high risk of disease after infection. *N. perurans* is associated with a specific disease with characteristic tissue lesions. This association has been consistently found across different populations and studies. The pathogen is detected in the gills prior to development of disease, and there is a dose-response relationship between agent load and disease and pathology. Lastly, ample experimental evidence for causality has been provided. Thus, Hill's criteria for causality are satisfied for this agent (Table 3).

Several studies have shown that salmon gill poxvirus is the cause of salmon gill poxvirus disease (SGPVD) in the freshwater phase of production (Gjessing et al., 2020; Gjessing et al., 2015; Nylund et al., 2008; Thoen et al., 2020), but the role of the virus in cases of gill disease after sea transfer remains inconclusive. In the current study, there was no association between load of SGPV and the extent of gill tissue with hyperplasia, hyperplasia and inflammation, vascular lesions, or tissue with overlap of these lesions. There was a positive association between SGPV load and epithelial cell necrosis or apoptosis, where fish with moderate loads of SGPV were 21,6 times more likely to have this lesion compared to fish negative for SGPV. While SGPV was detected

in all fish-groups, the majority of positive gills contained low amounts of viral genetic material.

Similarly, a prevalence ranging from 10-67%, overall high Ct-values for SGPV, but no epithelial necrosis or apoptosis was found among five Norwegian sea farms with outbreaks of gill disease (Gjessing et al., 2019). In a cohort study from Scotland, prevalence ranged from 0-100% and moderate to low loads of SGPV were detected. The SGPV load was not associated with increases in histological gill scores or extent of gill pathology. Lesions typical of SGPV were not detected in the sea phase of production (Herrero, Rodger, et al., 2022). Downes et al. (2018) performed a cohort study following a single farm and fish-group in Ireland. In this study SGPV prevalence ranged from 0-100% with a low SGPV load detected at all sampling points. SGPV load was not associated with the variation of histological gill scores. In contrast to the former studies, Nylund et al. (2008) described losses close to 80% at two marine farms with PGD and SGPV-infection, however *N. perurans* and *Candidatus* Piscichlamydia salmonis were also detected in all tested fish.

Because SGPV can disrupt the epithelial barrier and lead to downregulation of host immune genes, it was initially suspected that the virus was a primary pathogen that could pave the way and make fish more susceptible to infection and disease caused by other gill pathogens (Gjessing et al., 2020; Gjessing et al., 2017). Thoen et al. (2020) experimentally induced disease and characteristic histopathology lesions in fish intra-peritoneally injected with hydrocortisone and exposed to dead SGPV infected fish. Exposed fish without hydrocortisone injections became infected but did not develop clinical signs or mortality after exposure. The fish that developed disease and pathology had higher loads of the virus in gill tissue, compared to fish without lesion and clinical signs. Gene expression analysis of these fish indicated suppression of gill mucosal immune response and a late triggering of the systemic immune response in hydrocortisone treated fish (Amundsen et al., 2021).

Attempts to culture SGPV have so far been unsuccessful. However, the successful induction of disease in the cohabitation study means that Hill's criteria of temporality and experimental evidence are satisfied (Table 3). The criteria of specificity, consistency and dose-response are also met. SGPV is linked to a specific disease. Mortality and characteristic acute lesions of apoptotic epithelial cells are associated with high loads of the virus and detection of virions with transmission electron microscopy, ISH and IHC in affected cells (Gjessing et al., 2020; Gjessing et al., 2021;

Gjessing et al., 2017; Gjessing et al., 2015; Nylund et al., 2008; Thoen et al., 2020). It remains unclear what determines if infections lead to gill disease and pathology. Anecdotal evidence from the field has shown that SGPV infection is common in both freshwater and seawater sites, but SGPVD is seen in the freshwater phase and stressful events often occur prior to disease outbreaks. This observation agrees with results from the experimental study, which suggest that stress and immunosuppression is necessary for development of salmon gill poxvirus disease (Amundsen et al., 2021; Thoen et al., 2020).

Hill's criteria of causality	Questions for gill pathogens	*N. per*	SGPV	*D. lep*	*Ca*. B. cys
Strength of association	Is there a high risk of disease after infection? Is there a strong association between infection and gill pathology?	Y Y	N Y	N I	N I
Consistency	Has an association between agent and gill disease been found in different populations under different circumstances?	Y	Y	I	I
Specificity	Is the agent associated with only one clinical syndrome? Is the agent associated with a limited number of (characteristic) gill lesions?	Y Y	Y Y	Y I	Y I
Temporality	Does infection precede gill disease or does increases in pathogen load precede pathology?	Y	Y	I	I
Biologic gradient /Dose-response	Is there a dose-response effect in which a high pathogen load is associated with more severe gill pathology or clinical signs of disease?	Y	Y	I	I
Experimental evidence	Have experimental studies shown gill disease development following infection?	Y	Y	U	U

Table 3. Hill's criteria of causality and gill disease. Questions adapted to selected infectious agents involved in gill disease of farmed Atlantic salmon. The table provide a very simplified overview of the current knowledge status for each microorganism. Y indicate that evidence have been presented, U indicate unknown or that information is missing, N indicate that evidence against the claim exist, I indicate that evidence

presented so far is inconclusive or conflicting. Criteria based on prior beliefs are excluded from the table.

In our cohort study, which was performed from 2018 to 2020, no consistent covariation between the extent of gill pathology and prevalence or load of *Ca.* B. cysticola and *D. lepeophtherii* was observed. We did not find any association between gill pathology, gill-related mortality, and load of *Ca.* B. cysticola or *D. lepeophtherii* (Paper I). The only exception to this was a positive association between the *Ca.* B. cysticola load and epithelial cell necrosis or apoptosis of the superficial lamellar epithelium. Lesions that have been attributed to *D. lepeophtherii* and *Ca.* B. cysticola were observed in the sampled fish, but the type of such lesions and their presence were not consistently recorded or quantified. While burdens of *Ca.* B. cysticola ranged from high to low, very few of the sampled fish had a high burden of *D. lepeophtherii* in the gills.

Considering all the studies and knowledge about *Ca.* B. cysticola and *D. lepeophtherii* accumulated so far, results do not consistently show strong association between infection and disease, or infection and gill pathology (Table 3). The risk of disease after infection is unknown but cannot be high as both infections and observable epitheliocysts repeatedly have been found in fish without clinical signs of gill disease or lesions reported to be associated with the agents (Gjessing et al., 2021; Gunnarsson et al., 2017; Herrero, Rodger, et al., 2022; Nylund et al., 2011; Steinum et al., 2015; Steinum et al., 2010).

Both agents satisfy the criteria of specificity in that they are associated primarily with gill disease. If subclinical infection with an infectious agent is common in a population, diagnosis of disease caused by that infectious agent will often rely on a combination of pathogen detection, clinical signs, and necropsy findings. Consistently finding high amounts of pathogen genetic material in sick animals and identifying the suspected agent within characteristic or at least similar tissue lesions using methods like immunohistochemistry or in situ hybridisation supports a role for the pathogen in disease development (Segalés, 2012). The repeated finding of ballooning degenerative cells, necrosis and "microvesicles" with pigment in *D. lepeophtherii* infected gills indicate that this lesion is characteristic for *D. lepeophtherii* (Herrero, Palenzuela, et al., 2022; Nylund et al., 2011; Weli et al., 2017). *D. lepeophtherii* spores and organisms have repeatedly been detected within gill lesions using *in situ* hybridization and calcofluor white staining (Gjessing et al., 2021; Herrero,

Palenzuela, et al., 2022; Weli et al., 2017). The number of ISH-labelled *D. lepeophtherii* organisms and Ct-levels correlated with the severity of "microvesicles" and necrosis in one study, supporting the notion that these lesions might be characteristic and dose-dependent (Herrero, Palenzuela, et al., 2022). However, each of these studies include relatively few samples and the organism has also been detected in areas of the gill without such lesions (Gjessing et al., 2021; Herrero, Palenzuela, et al., 2022). Ballooning degenerative cells have been observed without detection of intralesional *D. lepeophtherii* organisms (Gjessing et al., 2021) and similar lesions have been observed in gills negative for *D. lepeophtherii* by PCR-analysis (personal communication H. Wisløff).

Lesions reported to be associated with epitheliocysts or *Ca.* B. cysticola infection, if any, appear to vary more across cases and studies (Gjessing et al., 2021; Gjessing et al., 2019; Mitchell et al., 2013; Rodger, Murphy, et al., 2011; Steinum et al., 2010). Gjessing et al. (2021; 2019) exclusively examined fish with suspected gill disease and stated that pustules, accumulation of inflammatory cells within hyperplastic epithelium, subepithelial necrosis and inflammation were associated with *Ca.* B. cysticola yet demonstrated with *in situ* hybridization that bacteria were found in areas of the gills both with and without such lesions. No details about a subepithelial neutrophilic inflammation believed to be associated with *Ca.* B. cysticola infection in several outbreaks of gill disease in Norway have been published so far. These lesions were observed in fish from freshwater and seawater, no other infectious agents were detected in submitted gills, and moderate to high loads of *Ca.* B. cysticola were found in the gill tissue (E. Thoen personal communication). However, it should be noted that most infectious agents are not associated with pathognomonic lesions and that Hill's specificity criteria have been criticised as it became increasingly recognized that it is common for infectious agents and environmental insults to be associated with or cause more than one disease.

In this discussion, dose-response effect refers to whether there is an association between load of *Ca.* B. cysticola or *D. lepeophtherii* and extent of pathology or disease. No studies reporting effects of exposing fish to different concentrations of the agents have been published. So far, it has not been conclusively established that increases in *D. lepeophtherii* and *Ca.* B. cysticola load precede subsequent development of gill disease or pathology. No consistent covariation between the amount of these agents and gill pathology or gill-related mortality were observed in our study. Higher *D. lepeophtherii* load, higher prevalence, and number of epitheliocysts was found in

proliferative gill inflammation (PGI) affected gills compared to non-affected gills in a Norwegian study including 12 sites repeatedly sampled in 2004 (Steinum et al., 2010). However, only the last sample set collected was analysed if fish-groups did not develop clinical signs of gill disease, and PCR-results for *D. lepeophtherii* were not included in the serial data available from 5 of the 12 farms. No clear covariation between *D. lepeophtherii* or *Ca.* B. cysticola load and histological gill scores or gill-related mortality were seen in the Scottish or Irish cohort studies (Downes et al., 2018; Herrero, Rodger, et al., 2022).

The different studies yield conflicting results on whether there is an association between microbe load and pathology or disease for both agents. Our study and the two smaller cohort studies from Scotland and Ireland published after *N. perurans* became endemic did not show an association between *Ca.* B. cysticola and *D. lepeophtherii* and the severity of gill disease (Downes et al., 2018; Herrero, Rodger, et al., 2022). In a Norwegian cohort study including 6 sites sampled 4 times in 2011-2012, higher *Ca.* B. cysticola loads were not associated with a clinical diagnosis of gill disease and presumed gill-related mortality. In contrast, *D. lepeophtherii* load was found to be positively associated with both clinical gill disease and runting (Gunnarsson et al., 2017). Nylund et. al. (2010) repeatedly sampled fish populations at two Norwegian farms suffering losses from an unidentified disease and identified *D. lepeophtherii* in all the sampled fish. Gill pathology was also described, but whether the extent of gill lesions varied with varying Ct-levels of the agent or over time were not reported, nor were fish tested for other potential pathogens.

Case reports have described outbreaks of gill disease and lesions associated with *D. lepeophtherii* in two individual sites in Norway and Scotland (Matthews et al., 2013; Weli et al., 2017). In a survey including 55 Norwegian farms from along the entire coastline, *D. lepeophtherii* was detected in 45 farms, 16 of which had issues with PGI. Mean *D. lepeophtherii* levels were significantly higher in fish from farms with a PGI diagnosis compared to fish from farms with other diagnoses (Nylund et al., 2011). Similarly, a study using archival diagnostic material from 2005, 2008 and 2009 from Norwegian farms with and without a diagnosis PGI found higher amount of *D. lepeophtherii* genetic material in fish with PGI compared to fish with other disease diagnoses (Hamadi, 2011). Mitchell et al. (2013) reported higher loads of *Ca.* B. cysticola in fish with more severe PGI grades in a study based on archival research material from Norway and Ireland including fish with and without a PGI diagnosis sampled from 2004 to 2010.

The results of different studies are not consistent, although there are a greater number of studies supporting an association between *D. lepeophtherii* and gill disease and pathology (Gjessing et al., 2021; Gjessing et al., 2019; Gunnarsson et al., 2017; Hamadi, 2011; Herrero, Palenzuela, et al., 2022; Matthews et al., 2013; Nylund et al., 2011; Nylund et al., 2010; Steinum et al., 2010; Weli et al., 2017), compared to *Ca.* B. cysticola (Gjessing et al., 2021; Gjessing et al., 2019; Mitchell et al., 2013). The conflicting results may be related to variation in microbe loads and disease status among the sampled fish, criteria used to diagnose gill disease and assess the gill tissue, study design, time of sample collection and more. Many of the studies described above are case-series, case-reports, or case-control studies, including fish-groups sampled only once and usually because of suspicions or clinical signs of gill disease or other diseases at the site. However, if fish are sampled when they are already sick it is not possible to establish that an increase in *Ca.* B. cysticola or *D. lepeophtherii* load occurred prior to development of disease and pathology. The inclusion of cases from a single site or few cases obtained from archival diagnostic and research material used in the ISH-studies so far means that these cases may differ from cases with the same disease that were not sampled. For this reason, more cases should be examined to ensure results are representative for the larger population (Caswell et al., 2018a). If the aim is to determine whether a highly prevalent infectious agent is associated with pathology and disease, only including cases with gill disease leads to risk of spurious associations and erroneous interpretations.

Our study is the largest cohort study published so far, yet our ability to draw conclusions are limited by the relatively few fish-groups included and the lack of severe gill disease at most of the study sites. Nonetheless, a cohort study provides stronger evidence of causality compared to other observational or descriptive studies (Caruana et al., 2015). This is because events are measured in chronological order in cohort studies. In contrast outcome and exposure are recorded at the same time for cross-sectional studies, and case-control studies compare groups retrospectively (Mann, 2003). The hierarchy of evidence (Figure 13) illustrates the levels of evidence associated with different study designs (Pfeiffer, 2010). The evidence pyramid focuses on the internal validity (risk of bias) with different study designs and is a very simplified way to present a complex reality (Murad et al., 2016; Rothman, 2014). Several ways to rank levels of evidence have been suggested (Burns et al., 2011; Murad et al., 2016), and how well a study is designed will determine how valid the results are (Concato et al., 2000; Rothman, 2014). Nonetheless, a well-designed

randomized controlled challenge study in a laboratory environment demonstrating disease and pathology in infected fish would have provided strong evidence of causality. Randomized controlled field trials challenging fish with ubiquitous infectious agents are unlikely to be successful because the control groups have a high risk of becoming infected.
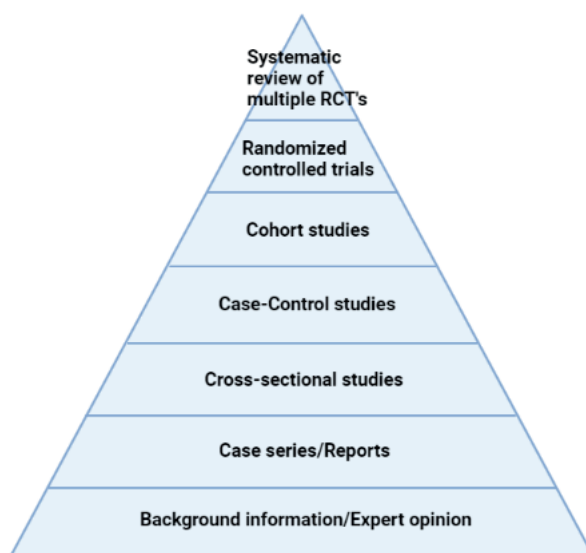
Figure 13. The hierarchy of evidence*. Study designs are ranked according to the level of evidence they provide from high to low. There are several ways to rank the levels of evidence associated with different study designs, and the appropriate ranking may vary depending on the research questions asked (Figure adapted from Pfeiffer (2010) and created in BioRender.com).*

The inability to culture, and subsequently infect naïve salmon with *Desmozoon lepeophtherii* and *Candidatus* Branchiomonas cysticola means that Koch's postulates have not been fulfilled for these agents. So far, no reports of experimental *D. lepeophtherii* infection of salmon have been published. In the single cohabitation challenge study infecting fish with *Ca*. B. cysticola, infected fish developed moderate lamellar epithelial hyperplasia and subepithelial inflammation but no clinical signs of gill disease. Because fish were concurrently infected with SGPV and *Ca.* Piscichlamydia salmonis it is not possible to determine if the observed lesions were caused by a specific infectious agent (Wiik-Nielsen et al., 2017).

To conclude, neither Koch's postulates nor Hill's criteria of causality are satisfied for *Ca.* B. cysticola or *D. lepeophtherii* (Table 4). This does not mean that these agents cannot or do not cause gill disease, but that firm evidence of causality has not yet been presented. The widespread *D. lepeophtherii* and *Ca.* B. cysticola infections in apparently healthy Atlantic salmon indicates that predisposing factors are necessary for these agents to cause gill lesions and gill disease and/or a variation in virulence and pathogenicity within these species.

Whether an infectious agent can cause gill disease and whether it is an important cause of gill disease in sea farmed salmon are two separate issues. Gill disease and infections with microorganisms implicated in gill disease are not notifiable in Norway (Dyrehelseforskriften, 2022, Kapittel II). No reliable official data on the number of sea sites with detected gill infections or disease outbreaks exist (Sommerset et al., 2022). To understand the relative importance of different agents in development of gill disease at sea it is necessary to have information on the sequence of events. Thus, the last question can only be answered by examination of the real-life situation, preferably by well-designed, large cohort studies. As shown by the discussion in the previous paragraphs, very few cohort studies on gill health in farmed Atlantic salmon exist, and our understanding of the impact of different infectious agents remain limited.

In sea farmed salmon SGPV can lead to gill pathology and lamellar epithelial apoptosis, but most studies describe mild epithelial pathology (Paper I; Gjessing et al., 2021; Gjessing et al., 2017; Nylund et al., 2008). In addition, most of the studies performed so far, including our study, have not found an association between SGPV and other types of gill pathology in sea farmed salmon (Downes et al., 2018; Gjessing et al., 2019; Herrero, Rodger, et al., 2022). The Norwegian cohort study from 2011-2012 reported that *D. lepeophtherii* loads, but not *Ca.* B. cysticola loads, were associated with gill disease (Gunnarsson et al., 2017). *N. perurans* were only sporadically detected and with low loads. In contrast, *Ca.* B. cysticola or *D. lepeophtherii* were not associated with extent of gill pathology in our study, nor in the Irish and Scottish cohort studies (Paper I; Downes et al., 2018; Herrero, Rodger, et al., 2022). In sum, the three cohort studies performed after AGD became endemic in Northern Europe found that *N. perurans* was the most important driver of gill pathology in the examined cohorts (Paper I; Downes et al., 2018; Herrero, Rodger, et al., 2022).

Our cohort study further confirmed that coinfections with multiple putative gill pathogens are to be expected in sea farmed Atlantic salmon in Western Norway. All fish-groups became persistently infected with *D. lepeophtherii* and *Ca.* B. cysticola, with seasonal infections by SGPV and *N. perurans*, though few groups developed severe gill pathology or associated mortality. Because of the high prevalence of these infections, it was not possible to determine if, for example, outcomes of *N. perurans* infection were worse in fish-groups with pre-existing or concurrent *D. lepeophtherii* and *Ca.* B. cysticola infection. There was low correlation between pathogen load of the different agents, with exception of a moderate correlation between pathogen load for *D. lepeophtherii* and *N. perurans.* The prevalence and pathogen load of the four pathogens largely followed different patterns. No blooms or swarms of plankton were detected in the study period, and no increase in pathogen load or AGD prevalence was evident following peaks in total phytoplankton levels detected at two of the study sites.

An association between *Ca.* B. cysticola or *D. lepeophtherii* and PGI was reported in earlier studies (Hamadi, 2011; Mitchell et al., 2013; Nylund et al., 2011; Steinum et al., 2010). Because the criteria for this condition include co-localization of inflammation, epithelial hyperplasia, circulatory disturbances and cell death of lamellar epithelium, these findings could be interpreted to suggest that the above agents are possible causes of vascular lesions in the gills. However, in the current study there was low or no correlation between pathogen load for any of the four agents and the extent of gill vascular lesions or overlap of hyperplastic and vascular lesions. Chronic inflammation of the filaments, lamellar epithelial hyperplasia and vascular lesions observed in two fish-groups were not associated with higher loads of pathogens compared to other fish-groups. These observations suggest that factors other than the gill pathogens included in this study could be involved in the development of these lesions. This could include infectious agents not tested for in this study, environmental, managerial and host factors (Figure 7). The current study further showed that pasteurellosis has become a cause of gill lesions in Atlantic salmon in Western Norway (Legård & Strøm, 2020; Sommerset et al., 2022). It is possible that lamellar thrombi and vascular lesions are related to concurrent and/or previous systemic bacterial infection in some cases, even when bacteria are not observed in the gill tissue.

5.4.3 Is variation in levels of phytoplankton and gelatinous zooplankton associated with gill disease in Norwegian farmed Atlantic salmon?

There was no discernible impact of zoo- and phytoplankton on any of the gill outcomes or the total mortality in our cohort study (Paper I). Further, generally low concentrations of plankton and jellyfish were detected. *Pseudo-nitzschia* spp. and other diatoms were the dominating type of phytoplankton observed. *Pseudo-nitzschia* species are not reported to be associated with fish mortality or disease in Norwegian waters (Karlson et al., 2021). It cannot be ruled out that brief and transient phytoplankton and zooplankton blooms might have been missed because of the sampling regime. Furthermore, farms were asked to note observations of larger jellyfish when collecting water samples or if blooms occurred. The lack of reported sightings of jellyfish may reflect low levels of jellyfish at study sites but may also suggest that jellyfish observations were not consistently recorded and reported by site staff. Nonetheless, the lack of association between plankton levels and gill pathology and mortality in our cohort study is likely related to the lack of a substantial plankton and jellyfish bloom at any of the sites during the study period. Because of this, we cannot draw any conclusions on whether phytoplankton and gelatinous zooplankton can cause gill disease and pathology based on our material.

To what extent Hill's criteria of causality are satisfied for phytoplankton and gelatinous zooplankton and gill disease or gill pathology varies for the different species of plankton. However, a detailed discussion of Hill's criteria and plankton is not included because our study did not generate any useful information about this topic. To the best of our knowledge there has not been published any observational studies exploring the impact of plankton blooms on gill health of Norwegian farmed Atlantic salmon prior to our cohort study. The lack of harmful algal blooms and jellyfish blooms in the study period indicate that these are events are not very common in the locations included in the study (Paper I). Nonetheless, our findings do not mean that gill and overall health of Norwegian sea farmed Atlantic salmon is not affected phytoplankton and gelatinous zooplankton. Fish disease and mortality associated with gelatinous zooplankton and phytoplankton in Norway appear to be sporadic events (Båmstedt et al., 2012; Hatlem, 2011; Hellberg et al., 2003; Karlson et al., 2021; Rodger, Henry, et al., 2011; Sommerset et al., 2022). However, the lacking monitoring of plankton levels could lead to blooms going undetected and the impact such events may be underestimated (Kintner & Brierly, 2019). Further work is

required to explore this and to understand if and how HABs and cnidarians cause gill injury and disease alone or through interactions with microorganisms and other insults.

5.4.4   Does *in situ* net cleaning impact gill health?

Our field trial examining effects of *in situ* net cleaning (Paper II) suggest that exposure to biofouling debris during *in situ* net cleaning of moderately fouled net pens contribute to development of thrombi/subacute vascular lesions in the gills of farmed Atlantic salmon. This is the first study to show that net cleaning as performed in a commercial setting may have an impact on gill health. However, the negative impact of *in situ* net cleaning on gill health was small and short lived, and no difference in the prevalence of lamellar thrombi was evident at eight days after net cleaning. Very few lesions were observed in the gills both before and after net washing, consistent with overall good gill health. The net cleaning event was not associated with changes in daily mortalities, specific feed rate (SFR) or specific daily growth rate (SGR) when compared to the month prior to net cleaning. No moribund fish or fish with overt signs of gill disease were observed after net cleaning.

There are relatively few studies examining the impact of cleaning debris exposure, and the studies that have been performed, including ours, have limitations. The studies examining effect of net cleaning debris on gill health of Atlantic salmon are summarized in Table 4. Both field studies include few farms and fish-groups, which makes generalizability of the results difficult. Further, the low to moderate biofouling in our study may not have been representative for fouling situation at most farms. This means that the concentration of fouling particles in the water could have been too low to cause gill injuries, which may occur at the higher particle concentrations following washing of heavily fouled nets.

Reference	Type of study	Fish-groups n per group/ sampling point Total n	Exposure concentration	Exposure time/ cleaning time	Biofouling species	Findings
Baxter et al. 2012	Laboratory study	6 (tanks) n=5 n=185	Unknown (not clearly stated)	11 hours	E. larynx	No difference between exposed and control fish. Haemorrhage, epithelial necrosis, and sloughing was found in all groups post-exposure
Bloecher et al. 2018	Laboratory study	8 (tanks) n=2-5 n=272	10 000 polyps per m^3	3 hours	E. larynx	Increased prevalence and severity of lamellar thrombosis Higher non-specific gross scores No effect of hydroid exposure in infection rates of N. perurans or disease progression of AGD
Napsøy, 2020	Field study, commercial sites	3 (pens/ sites) n=8-30 n=188	Maximum turbidity of 2.0 to 4.5 FTU and 5-22 % sediments in water samples across study pens	3 to 4 hours	Multiple, including E. larynx	No difference between pre- and post-cleaning gill samples No significant increase in prevalence of gill pathogens after cleaning. (AGD present prior to net cleaning).
Østevik et al. 2021 Paper II	Field study, commercial site	3 (pens) n=30 n =270	Unknown (low to moderate biofouling)	80 minutes to 2 hours	Unknown, (possibly including E. larynx)	Increased prevalence of lamellar thrombi/subacute vascular lesions

Table 4. Studies on biofouling and gill health of Atlantic salmon.

A recent field trial of *in situ* net cleaning including three fish-groups and commercial sea sites showed no clear difference in gill lesions between different sampling points (Napsøy, 2020). The lack of systematic registration of histologic lesions and the presence of amoebic gill disease in two of the three sites sampled may have limited

the ability to detect differences between fish sampled before and after cleaning. An increased extent and prevalence of lamellar thrombi was found in the gills of Atlantic salmon after exposing fish to *E. larynx* in a laboratory experiment (Bloecher et al., 2018). No difference was found between exposed and control fish in another experimental study. Epithelial necrosis and haemorrhage described in both groups were suggested to be caused by biofouling fragments entering the water circulation and control tanks (Baxter et al., 2012). However, the figures illustrating purported histological gill lesions in the above mentioned paper are not convincing in illustrating tissue pathology and does not support the findings of haemorrhage and epithelial necrosis reported.

It is unclear to what extent exposure to net cleaning debris in the laboratory mimics the actual situation during net washing in the field. Though Bloecher at al. (2018) used exposure times and concentrations of biofouling organisms aimed to be similar to the real-life situation at Norwegian sea farms, the measurements of turbidity and percent sediment in the water samples in the field study from 2020 (Napsøy, 2020) showed that the amount of debris in the water can vary across sites and over time during the net cleaning process. If currents continuously dilute and carry debris away from the pen, the time and concentration of biofouling material fish are exposed to may be less than in the laboratory studies performed so far.

The current knowledge suggests that exposure to net cleaning debris can cause increased prevalence and severity of lamellar thrombosis in the gills of Atlantic salmon. However, Hill's criteria for causality are currently not satisfied for *in situ* net cleaning and gill injuries. A strong association between exposure to biofouling and gill pathology has yet to be established. The risk of gill injuries after exposure to cleaning debris is unknown. Whether there is a dose-response effect, in which a certain concentration and length of exposure to biofouling particles are necessary to cause gill injuries, has not been explored. While an increase in severity and/or prevalence of lamellar thrombi have been reported after biofouling exposure in two studies, such lesions are not consistently observed in all experimental or field studies (Paper II; Baxter et al., 2012; Bloecher et al., 2018; Napsøy, 2020). Thus, both the type of lesions and whether exposure leads to gill lesions vary across studies. Further, it is not established if *in situ* net cleaning can lead to clinical gill disease of farmed salmon in a commercial setting. No studies have examined the potential health effects of repeated net cleaning events. No evidence presented so far suggests that net cleaning leads to increased prevalence of gill infections or exacerbation of pre-existing

infectious gill disease (Bloecher et al., 2018; Napsøy, 2020). However, as shown above very little research has been done to explore the impact of *in situ* net cleaning on gill and overall fish health, and significant knowledge gaps remain. It is likely that differences in biofouling composition and concentration, anti-biofouling strategies and equipment, and site-specific factors like current velocity and direction, health status and size of the fish at time of net cleaning all determine if net cleaning negatively impacts gill health. Thus, further work is necessary to determine if and in what situations *in situ* net cleaning can lead to gill disease and pathology in Norwegian salmonid mariculture.

5.4.5   Does non-medicinal delousing impact gill health?

The results of our study (Paper III) indicate that thermal and mechanical delousing have a negative impact on gill health. An increase in the percentage and counts of lamella with vascular and hyperplastic lesions, and an increase in the number of fish with microorganisms observed in the gills were found after delousing. Gene expression analysis showed differential expression of genes involved in pathways of cell stress, inflammation, repair, and proliferation in the gill tissue after thermal delousing. RT-qPCR-analysis revealed increased load of *Ca*. B. cysticola after thermal delousing. The difference in distribution and characteristics of lesions observed after thermal and mechanical delousing may suggest different mechanisms behind the tissue injuries for the two treatments. The overall percentage of gill tissue with lesions was generally low, and the clinical impact of the observed lesions remains to be established. However, vascular, and hyperplastic lesions will reduce the amount of gill tissue available for oxygen exchange as thrombi will disrupt normal sinusoidal blood flow. In addition, hyperplastic lamellar epithelium increases the diffusion distance of gases from water to blood.

This is the first and only study examining the effects of thermal delousing on gill gene expression published so far. Comparison with other studies is therefore not possible. The increased expression HSP70, TNF-α, TGF-β, IL-1β, IL-4/13α, IL-10 and IFN-γ at 1 and 2 days after treatment indicate that thermal delousing induces responses related to cell stress, regulation, and promotion of inflammation, and repair. The continued upregulation of TNF-α, IL-1β and IL-4/13α at 8 and 9 days after treatment suggests that pro-inflammatory responses are maintained over the observation period. TNF-α

and IL-1β lead to endothelial activation and can have prothrombotic effects (Pircher et al., 2012; Yoshida et al., 2010), and a sustained inflammatory response could therefore be a factor in the increase of vascular lesions observed after thermal delousing. EGFR was upregulated at 1 and 2 days after treatment in two fish-groups. IL-4/13α, IL-4/13β2, IL-1β, TNF-α and EGFR have been shown to be upregulated in hyperplastic gill lesions in salmon with amoebic gill disease (Benedicenti et al., 2015; Bridle et al., 2006; Marcos-Lopez et al., 2018; Morrison et al., 2012; Morrison et al., 2007; Pennacchi et al., 2014). However, to what extent the hyperplastic responses in the current study are linked to upregulation of inflammatory cytokines or EGFR remains to be determined. There was a low correlation between the extent of microscopic gill lesions and fold change of gene expression, which was somewhat surprising as we expected to find a link between at least some genes and the degree of gill histopathology. Possible explanations for this could be that selected genes are not important for the development or progression of the lesions observed, differences in timing of differential gene expression and when lesions are evident and/or mismatch between mRNA and active protein levels in the tissue.

Our study is one of the few studies that has explored if thermal delousing can impact the prevalence and load of microorganisms in the gills of Atlantic salmon. The increased load of *Ca.* B. cysticola and increased prevalence of gill microorganisms and tissue lesions possibly associated with gill pathogens *Ca.* B. cysticola and *D. lepeophtherii* after delousing could represent an effect of the treatment. If so, it could be related to stress and increased cortisol levels leading to increased susceptibility to infection and reduced ability to limit proliferation of infectious agents (Erikson et al., 2018; Pickering & Pottinger, 1985), though consistent down-regulation of pro-inflammatory cytokines was not evident after treatment. It has been demonstrated that stressful events like thermal delousing and experimental warm water exposure lead to increased secretion of *Yersinia ruckeri* in Atlantic salmon with subclinical infection (Strand et al., 2021). In contrast to our findings a recent field study with a similar design reported no increases in the load of gill pathogens or other infectious agents after treatment (Kvåle, 2020).

The findings in our study are in contrast with an increasing number of laboratory studies and field trials reporting no clear increase in the severity of gill injuries after heated water exposure or thermal or mechanical delousing (Bentzen et al., 2018; Bui et al., 2022; Ellingsen & Moljord, 2019; Erikson et al., 2018; Grøntvedt & Kristensen, 2018; Grøntvedt et al., 2015; Kvåle, 2020; Mangor-Jensen et al., 2017; Moltumyr et al.,

2021; Moltumyr et al., 2022; Roth, 2016; Salvesen et al., 2021; Westgård et al., 2021). A significant increase in grossly visible gill haemorrhages was reported after FLS treatment (Gismervik et al., 2017). However, a later study of an improved FLS system found only mild gill haemorrhage and concluded that the new system significantly reduced the risk of gill haemorrhage (Grøntvedt & Kristensen, 2018). In a study of 332 thermal treatments in northern Norway higher mean scores, but not median scores, for gross gill haemorrhages were reported after treatments in 2018 and 2019, but not 2020 and 2021 (Lund et al., 2022). Gill aneurysms (described by Gismervik et al. as bleeding), haemorrhage and necrosis have been reported after warm water exposure in the laboratory or thermal delousing in the field in two other studies (Gismervik et al., 2019; Jørgensen & Rød, 2019).

The discrepancies between our findings and the findings of other researchers could have several reasons. There could be differences between fish-groups, environmental factors, delousing methods, and equipment used explaining why we find gill injuries after delousing when others do not. Gross scoring of external tissues (skin, gill, fins, and eyes) and percent mortality after treatment are the main parameters used to assess effect of delousing in most previous field studies. As discussed in section 5.3.5.2 gill lesions may go undetected during gross examination. Quantitative, or semi-quantitative microscopic assessment of gill tissue was not performed in most of the previous studies examining effects of mechanical delousing nor in most field trials of thermal delousing (Bentzen et al., 2018; Erikson et al., 2018; Gismervik et al., 2017; Grøntvedt & Kristensen, 2018; Grøntvedt et al., 2015; Nilsen et al., 2010; Roth, 2016; Salvesen et al., 2021). Lastly, the histopathological assessment performed in the current study gives a high degree of detail and because of this, smaller increases in extent of tissue damage can be detected compared to an ordinal four- or five-tier scoring system (Adams et al., 2004; Birkebak & Mann, 2019; Meyerholz & Beck, 2018).

Exposing fish to warm water in a laboratory is very useful for studying the effects of heated water exposure on fish behaviour, health, and welfare. Some of these studies have been criticised for being unrealistic, using too long exposure times, too high water temperature or delta water temperature, or too small fish, possibly overestimating the negative effects of thermal treatment (Gillund & Nygaard, 2021; Gismervik et al., 2019; Moltumyr et al., 2021; Moltumyr et al., 2022; Nilsson et al., 2019). However, laboratory experiments can also be criticised for not accurately mimicking the total impact of a treatment event as it occurs at commercial sea sites.

These studies may therefore also underestimate the negative effects of delousing on treated fish. Despite lack of injuries or relatively minor injuries reported in most laboratory studies, mechanical injuries related to delousing treatments are reported as among the most important causes of mortality and reduced fish welfare by Norwegian fish health personnel (Sommerset et al., 2022). This underscores that observational studies examining real-life situations are necessary to understand the impact of NMMs on fish health. Nonetheless, the lack of gill pathology in most laboratory studies suggest that exposing fish to warm water for 30 seconds does not directly cause gill injuries. Results of recent studies suggest that several of the injuries reported after thermal delousing may be the result of trauma sustained as fish try to escape the heated water (Moltumyr et al., 2021; Moltumyr et al., 2022; Nilsson et al., 2019), and fish sedated during treatment have been shown to have less behavioural responses, less injuries and lower post-treatment mortality (Folkedal et al., 2021; Lund et al., 2022).

In our study it is not possible to determine to what extent the lesions and differential gene expression in the gill tissue are caused by the delousing per se relative to the impact of crowding and pumping of the fish. However, crowding, pumping and movement of fish through treatment units are integral parts of NMM as they are currently performed by the industry. Even if injuries or mortality observed after delousing are not directly caused by the therapeutic intervention (i. e. warm water or flushing), the treatment is still the reason fish sustained injuries or died. As outlined above, gill lesions and changes in prevalence and load of gill microorganisms are not a consistent finding in studies on thermal and mechanical delousing. Further, while the vascular injures and epithelial hyperplasia in the current study leads to fewer lamella available for gas exchange it is not clear that this reduction will have an impact on the clinical condition of the fish. No effect on gill health was reported in two laboratory trials repeatedly exposing fish to heated water (Bui et al., 2022; Moltumyr et al., 2022). However, if non-medicinal delousing in the field can have cumulative, long-term, and indirect effects of on gill health remains largely unknown. For instance, whether non-medicinal delousing impacts development and outcomes of infectious gill disease have not been explored.

Ignoring gills for a moment, the current field study (Paper III) and the cohort dataset (Paper I) show that non-medicinal delousing is associated with increased mortality and is among the most important causes of death and loss in the sea phase of production (Østevik et al., 2022). This has also been shown repeatedly in several

studies (Bang Jensen et al., 2020; Lund et al., 2022; Oliveira et al., 2021; Overton et al., 2018; Persson et al., 2021; Sviland Walde et al., 2021). Fish show behavioural responses indicative of pain during warm water exposure (Bui et al., 2022; Folkedal et al., 2021; Moltumyr et al., 2021; Moltumyr et al., 2022; Nilsson et al., 2019). Reduced growth rate after treatment has been reported both in laboratory and field studies (Bui et al., 2022; Moltumyr et al., 2022; Walde et al., 2022). A wide range of injuries in different organ systems have been shown or suggested to be associated with warm water exposure and/or non-medicinal delousing (Bentzen et al., 2018; Bui et al., 2022; Erikson et al., 2018; Gismervik et al., 2019; Gismervik et al., 2017; Hjeltnes et al., 2018; Jørgensen & Rød, 2019; Lund et al., 2022; Moltumyr et al., 2021; Moltumyr et al., 2022; Nilsen et al., 2010; Poppe et al., 2018; Poppe et al., 2021). It is unclear whether all the reported injuries are directly related to the treatment component of thermal or mechanical delousing or if injuries could be caused by handling, the behavioural response to treatment or in some cases are unrelated or pre-existing conditions. However, as death is the ultimate negative health outcome the increased mortality associated with treatments demonstrate that the non-medicinal delousing undoubtedly has a negative impact on fish health and welfare. A decrease in mortality associated with thermal delousing has been observed in recent years, likely due to improved procedures and equipment with experience (Lund et al., 2022; Sviland Walde et al., 2021). Nevertheless, it is imperative to continue to improve these methods and to develop alternative ways to prevent, control and treat sea lice infestations.

5.5   Conclusions

The work presented in this thesis has added new knowledge about gill disease and infectious agents, managerial and environmental factors which might be involved in development of gill disease and injury. At the outset of the study no cohort study including this many sites and fish-groups with repeated microscopic examination and PCR-analysis of gill tissues had been performed. Further, we showed that *in situ* net cleaning, thermal and mechanical delousing may impact gill health by performing systematic and detailed histological examination, PCR-analysis for gill pathogens and gene expression analysis on collected gill tissue. To summarize:

- We have described the temporal development of prevalence and load of *N. perurans*, *Ca.* B. cysticola, *D. lepeophtherii* and SGPV in 16 cohorts of sea farmed Atlantic salmon (Paper I).
- We have shown how variation in pathogen prevalence and load of *N. perurans*, *Ca.* B. cysticola, *D. lepeophtherii* and SGPV relates to gill health in these cohorts (Paper I).
- We have shown that the measured variation in levels of phytoplankton and gelatinous zooplankton had no impact on gill health in our cohorts (Paper I).
- We found an increased risk of lamellar thrombi after *in situ* net cleaning of moderately fouled pens (Paper II).
- We demonstrated that thermal and mechanical delousing performed in a commercial setting can lead to acute gill damage, differential gene expression, and changes in pathogen prevalence and load (Paper III).

5.6   Future perspectives

- To increase our understanding of which factors affect gill health we will perform further analysis to explore associations between pathogen levels, environmental factors, and non-medicinal delousing in our cohorts. Multiple regression models will be applied to control for several factors simultaneously.
- Cohabitation studies should be performed infecting fish with *Ca.* B. cysticola or *D. lepeophtherii* only to conclusively determine if these agents can cause disease. These studies should be performed with and without manipulation of immune status of the cohabitants.
- The impact of coinfections or secondary infections could be explored in controlled laboratory experiments. For instance, fish with and without *Ca.* B. cysticola or *D. lepeophtherii* infection could be infected with *N. perurans* to determine if these infections impact outcomes of *N. perurans* infection and development of amoebic gill disease.
- Large cohort studies including multiple sites and fish-groups should be performed to identify risk factors of gill disease and to determine the importance of coinfections, environmental insults, and health status of the fish for development of gill disease.

- If *in situ* net cleaning can have cumulative, long-term, and indirect effects of on gill health could be explored both by use of pre-existing production data and by field studies involving repeated sampling and laboratory analysis of tissues from sets of fish-groups subjected to multiple rounds of net cleaning. To determine how variation in the concentration of fouling particles and the species composition of the fouling community impacts gill health, laboratory experiments exposing fish to different concentrations and types of fouling material should be performed.
- Whether thermal or mechanical delousing have cumulative, long-term, and indirect effects of on gill health could also be explored. This could be performed by use of pre-existing production data and by field studies involving repeated sampling and laboratory analysis of tissues from sets of fish-groups subjected to multiple rounds of these exposures in the field.

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7 Enclosed papers I-III

I   A cohort study of gill infections, gill pathology and gill-related mortality in sea farmed Atlantic salmon (*Salmo salar* L.): Descriptive analysis

RESEARCH ARTICLE

A cohort study of gill infections, gill pathology and gill-related mortality in sea-farmed Atlantic salmon (*Salmo salar* L.): A descriptive analysis

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Abstract

Gill disease is an important cause of economic losses, fish mortality and reduced animal welfare in salmonid farming. We performed a prospective cohort study, following groups of Atlantic salmon in Western Norway with repeated sampling and data collection from the hatchery phase and throughout the 1st year at sea. The objective was to determine if variation in pathogen prevalence and load, and zoo- and phytoplankton levels had an impact on gill health. Further to describe the temporal development of pathogen prevalence and load, and gill pathology, and how these relate to each other. *Neoparamoeba perurans* appeared to be the most important cause of gill pathology. No consistent covariation and no or weak associations between the extent of gill pathology and prevalence and load of SGPV, *Ca.* B. cysticola and *D. lepeophtherii* were observed. At sea, *D. lepeophtherii* and *Ca.* B. cysticola persistently infected all fish groups. Fish groups negative for SGPV at sea transfer were infected at sea and fish groups tested negative before again testing positive. This is suggestive of horizontal transmission of infection at sea and may indicate that previous SGPV infection does not protect against reinfection. Coinfections with three or more putative gill pathogens were found in all fish groups and appear to be the norm in sea-farmed Atlantic salmon in Western Norway.

KEYWORDS
AGD, Atlantic salmon, *Ca.* B. cysticola, *D. lepeophtherii*, gill disease, SGPV

1 | INTRODUCTION

Gill disease is an important cause of economic losses, fish mortality and reduced animal welfare in the marine and freshwater phase of salmonid farming in Norway and across the world (Shinn et al., 2015; Sommerset, Bang Jensen, Bornø, Haukaas, & Brun, 2021). Reduced gill health may also impact outcomes of, and decisions about, management operations, such as non-medicinal delousing and transport,

because diseased fish might have reduced tolerance for handling and stress. Gill disease and gill injuries can be caused by infectious agents, environmental factors, management operations or a combination of these (Boerlage et al., 2020; Mitchell & Rodger, 2011; Rodger, Henry, & Mitchell, 2011). Gill disease has been classified as either simple or complex/multifactorial based on a presumption of single or multiple causes and/or infectious agents involved in the disease process (Gjessing, Thoen, Tengs, Skotheim, & Dale, 2017; Herrero,

Thompson, Ashby, Rodger, & Dagleish, 2018; Noguera et al., 2019). However, the exact cause(s) and pathogenesis of a considerable proportion of gill disease cases are unknown (Boerlage et al., 2020).

Microorganisms *Ca.* Branchiomonas cysticola, salmon gill poxvirus (SGPV), *Neoparamoeba perurans* (syn. *Paramoeba perurans*) and *Desmozoon lepeophtherii* (syn. *Paranucleospora theridion*) are commonly detected in gills of Norwegian sea-farmed Atlantic salmon (*Salmo salar* L), whereas the first two are also found during the freshwater phase of production (Downes et al., 2018; Gjessing et al., 2017; Gunnarsson et al., 2017; Nylund et al., 2008). *N. perurans* is the causative agent of amoebic gill disease (AGD) which has been causing endemic gill disease of variable severity in Norway since 2012 (Crosbie, Bridle, Cadoret, & Nowak, 2012; Mo, Hytterod, Olsen, & Hansen, 2015; Sommerset et al., 2021; Young, Crosbie, Adams, Nowak, & Morrison, 2007). SGPV infection can lead to salmon gill poxvirus disease (SGPVD) and high, acute mortality in hatcheries, though predisposing factors such as stress and immunosuppression may be necessary for disease development (Amundsen et al., 2021; Gjessing et al., 2015; Thoen et al., 2020). Whether SPGV is an important cause of clinical gill disease in the sea phase of production remains unclear, but SGPV infection and associated pathology have been reported in both fresh- and sea water (Gjessing et al., 2017; Gjessing et al., 2015; Nylund et al., 2008).

The understanding of the roles of *Ca.* Branchiomonas cysticola and *D. lepeophtherii* in the development of gill disease is limited by the lack of established challenge models and the fact that both agents are nearly ubiquitous in sea-farmed salmon in Northern Europe (Downes et al., 2018; Gunnarsson et al., 2017; Mitchell et al., 2013; Steinum et al., 2010). Whilst these agents are detected in salmon without gill disease, higher pathogen loads have been reported in diseased gills and the microorganisms have been detected in association with gill lesions using *in situ* hybridization (Gjessing et al., 2021; Mitchell et al., 2013; Weli et al., 2017).

Environmental factors such as harmful algal blooms (HABs) and jellyfish blooms can lead to significant fish mortality and gill injuries in sea-farmed salmon (Clinton, Ferrier, Martin, & Brierley, 2021; Rodger et al., 2010). However, HABs and jellyfish blooms causing clinical disease and mortality are relatively rarely reported and appear to be sporadic in Norwegian salmonid mariculture (Båmstedt, Fosså, Martinussen, & Fosshagen, 2012; Halsband et al., 2018; Karlson et al., 2021; Rodger et al., 2011; Smage et al., 2017). Monitoring of zoo- and phytoplankton levels is not commonly performed at Norwegian sea farms and whether zoo- and phytoplankton levels not associated with acute severe mortality can impact gill and fish health has not been explored.

Farmed Atlantic salmon will frequently be exposed to a range of potential insults and environmental factors during a production cycle at sea. Each might have an impact separately, but they can also coincide in time and interact to determine whether fish will develop clinical disease or mortality. Cohort studies are especially useful for evaluating the relationship between exposures and the development of disease, and to observe change over time. Further, the sequence of events can be established and it may be possible to identify and link events (like gill disease) to a particular exposure

(Caruana, Roman, Hernández-Sánchez, & Solli, 2015). However, relatively few longitudinal studies focusing on gill health including multiple sites and fish groups and with a parallel sampling of fish for histopathology and RT-qPCR have been performed.

In order to gain more knowledge about factors affecting the gill health of Atlantic salmon in Norwegian aquaculture, we performed a prospective cohort study, following 16 fish groups from 8 sea farms with repeated sampling and data collection from the hatchery phase and throughout the 1st year at sea. The objective of this study was to determine if variation in pathogen prevalence and load of *N. perurans*, *Ca.* B. cysticola, *D. lepeophtherii* and SGPV, and water concentration of jellyfish and phytoplankton had an impact on gill health as measured by gill-related mortality, gross gill scores and extent of tissue damage detected by histopathology. The secondary aim was to describe the temporal development of pathogen prevalence and load of *N. perurans*, *Ca.* B. cysticola, *D. lepeophtherii* and SGPV, observation of pathogens, and different types of gill pathology, and how these relate to each other. In this article, we report the descriptive analysis of these data and the associations between gross gill scores, extent of gill histopathology and pathogen load in individual fish.

2 | MATERIAL AND METHODS

2.1 | Study population

The main study unit was the 'fish group', defined as fish from the same hatchery transferred to sea at the same time and to the same cage. Parallel gross scores, histopathology and RT-qPCR results from individual fish additionally allowed for examination of the correlation between tissue lesions, pathogen prevalence and load using 'fish' as the study unit.

Four freshwater sites and eight seawater sites were included in the study. Fish groups were split into two pens at the same site at sea transfer, resulting in eight separate fish groups (A-H) during the freshwater phase and 16 fish groups (A1-H2) during the seawater phase of production (Figure 1). Freshwater sites were selected to include two sites using flow-through systems (FT), two using recirculating aquaculture systems (RAS), two sites with a history of gill disease (sites 3 and 4) and two sites without known gill issues (sites 1 and 2). From each freshwater site, one fish group sea transferred in autumn 2018 (S0) and one fish group sea transferred in spring 2019 (S1) were included. A winter signal was given at site 1 (12h light:12h dark for 5weeks, S0 fish only) and 3 (12h light:12h dark for 6weeks), and salinity was increased prior to sea transfer to facilitate smoltification at sites 2–4.

Sea sites were selected to include four sites with a history of problems with gill disease and gill-related mortality (sites A, C, D and G), whereas the remaining four other sites only had mild or no recorded historic gill-related mortality. Sea sites were in Western Norway and fish groups at each site were sea transferred at approximately the same time (A-D: August 2018, E-F: June 2019, G-H: April 2019). Sites were in fjords (A, B, D, G and H) with relatively low exposure or at sea (C, E and F) with moderate to high exposure to currents and waves.

FIGURE 1 Overview of freshwater sites ($n = 4$), sea sites ($n = 8$) and fish groups ($n = 16$). Stocking period (S0 = autumn stock/S1 = spring stock) and water treatment (flow through vs. RAS = circular arrow) is indicated
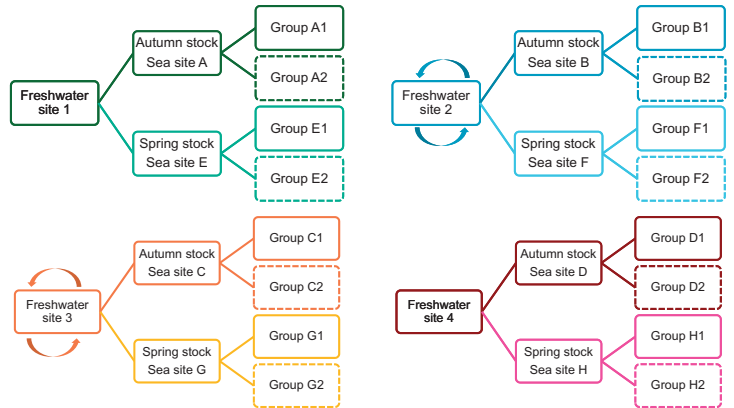
2.2 | Environmental data

Sea temperatures were recorded daily for each sea site, whereas salinity was only repeatedly recorded for sea sites B and C varying from >1 month to 1 week between recorded measurements. Measurements of salinity at the remaining sites were not available or very limited.

2.3 | Mortality data

Mortality data from all sites were retrieved through the management database Mercatus Farmer (ScaleAQ, Norway). The total number of fish dead per day (total mortality), the number of dead fish in each mortality category (cause-specific mortality) per day and the number of stocked fish per day were provided for each site and pen. Cause-specific mortality was generated by site staff daily assigning a likely cause of death based on gross examination of dead fish and knowledge of infectious disease and management events occurring at the site. Each fish was only assigned one mortality cause and if the classification of mortality to one category was not possible, mortalities were classified as 'unknown' or 'other'. Fish health personnel gave recommendations about classification and were further involved if there were any significant mortality events. Following visits by fish health personnel and/or results from laboratory analysis, the mortality categories could have been retrospectively adjusted. Results of RT-qPCR analysis performed as part of this study were made continually available to sites and may have been used when categorizing mortalities.

2.4 | Sampling of fish

Each fish group was followed prospectively with regular sampling in the freshwater (FW) and seawater (SW) phases, whereas regular gross gill scoring was performed in the sea phase only. In the freshwater phase, 20 to 30 fish per fish group were sampled 0 to 3 times for histopathology ($n = 350$) and RT-qPCR ($n = 378$) analysis. For a sample overview, see Table S1. Fish groups B and C were not

sampled during the FW phase. The time from sea transfer until first sampling at sea ranged from 20 to 84 days across fish groups and the time between each sampling ranged from 20 to 122 days. About 10 to 30 fish were sampled per fish group and at 6 to 10 time points at sea. Fish groups A2, B1, B2 and F1 were mixed with fish from other cages during summer and autumn 2019, whereas fish groups G1, G2, E1, E2 and F2 were split into smaller groups during spring, summer or autumn of 2020. During each splitting or mixing event, the pen with the most fish from the original fish group was designated the project pen and fish group and followed moving forward. Most mixing events occurred late in production, with only one sampling performed after mixing for three fish groups (A2, B1 and B2), whereas six sample sets were collected after mixing of fish group F1. After the mixing, 49% (B2), 64% (A2), 85% (B1) and 95% (F1) of the original fish group remained in the designated project pen.

At each sampling point, the aim was to sample up to 15 fish with clinical signs of disease and 15 presumed healthy fish per pen/tank, but no fish sampled during FW and very few fish sampled in SW showed clinical signs of disease ($n = 44$) and most were presumed healthy ($n = 2075$) or of unknown health status ($n = 1814$). In the freshwater phase, fish were sampled using a dip net. At the sea sites, fish in the net pens were crowded using feeding and a purse seine, and fish were selected from the seine with a dip net, as described in the standard operating procedure of the fish farms. Prior to tissue collection, fish were killed by placing them in anaesthetic bath until dead. Within 5 minutes after euthanasia, the second left gill arch was sampled for histology, whereas tissue samples from the third left gill arch were placed in RNAlater (Sigma-Aldrich) for RT-qPCR analysis. Gross gill scoring was performed on anaesthetized fish prior to tissue sampling as far as practically possible ($n = 3593$).

2.5 | Gross gill scoring

A gross gill score system based on the total area of abnormal tissue in the gill was adapted from a system developed by Fish Vet Group UK (personal communication Angela Ashby). Each left gill arch (both

TABLE 1 Gross score system. The lesions counting towards the score, score categories and the extent of gill tissue affected for each score category are outlined

Lesions	Score category	Gill tissue affected
White areas (presumed hyperplasia)	0	No abnormal gill tissue
Haemorrhages	1	<5% of gill tissue affected
Loss of gill tissue – shortened filaments	2	5–25% of gill tissue affected
Swollen, thickened gill tissue	3	25–50% of gill tissue affected
Yellow discolouration of gill tissue	4	50–75% of gill tissue affected
Fusion of filaments	5	75–100% of gill tissue affected
Necrosis (defined as grey or discoloured tissue and/or loss of normal tissue structure)		

surfaces) was scored separately on a scale from 0 to 5 and then, a mean gill score for all arches was calculated. For each scoring session and fish group, the median of the mean gill score and the proportion of fish with a mean gill score higher than 1 were calculated. Lesions counting towards the score and score categories are outlined in Table 1. Gross gill scoring of 20 fish per fish group was planned to be performed weekly during the sea phase, but between 6 and 50 fish were scored per time point and fish group, with scores available from 33 to 51 weeks across all fish groups ($n = 15,553$). Scorers included site staff, Mowi project participants, attending fish health personnel and veterinarians and varied from site to site and over time. The majority of scorers received a training session and example images of the different lesions to be scored and categories were provided to each site.

2.6 | Histopathology

Gills were fixed in 10% buffered formalin (4% formaldehyde, 0.08 M sodium phosphate, pH 7.0), processed routinely, and sectioned, stained with haematoxylin-eosin (HE) and scanned for histopathologic examination as previously described in Østevik et al. (2021). The study pathologists were 'blinded' regarding results of RT-qPCR-analysis, gross score or water analysis if available. Two different pathologists (HH and MA) examined samples from the freshwater phase, whereas a third pathologist (LØ) examined all samples from the sea phase.

A slightly modified two-step assessment protocol developed by the authors was used (Østevik et al., 2021). Briefly, first, the number of lamellae available for evaluation in each sample was estimated, and then, all affected lamella with hyperplasia or hyperplasia and inflammation, necrosis and vascular lesions (thrombi and aneurysms) were counted (Figure 2). For details and definitions of the type of lesions recorded, see File S1. These counts were used to calculate the estimated percent of gill tissue affected for each type of lesion. A total histology count was calculated by summarizing the number of lamellae with the lesions listed above and subtracting the number of lamellae with more than one lesion. The total percent affected tissue was then calculated by dividing the total histology count by the estimated lamellar count and multiplying by 100. Similarly, the percent of tissue affected by hyperplasia, vascular lesions or overlap

of vascular lesions, and hyperplasia was calculated for each fish. The presence or absence of the following lesions was recorded as 0 or 1 (dichotomous variables):

- Haemorrhage
- Epithelial cell necrosis or apoptosis
- Adhesion of lamella
- Lamellar oedema/'lifting'
- Deformed filaments
- Chronic inflammation of the filaments
- Amoebic gill disease (AGD)
- Foreign material present between lamella associated with tissue reaction

Any pathogens or microorganisms observed in or associated with the gill tissue were recorded as present or absent. For further work with the histopathology data on fish group level the median total percent affected tissue, median percent hyperplasia, median percent vascular lesions and median percent tissue with concurrent hyperplasia and vascular lesions per sampling point were used. In addition, the proportion of fish with the above lesions and pathogens as well as the proportion of fish with more than 5% total affected gill tissue was calculated for each fish group and sampling point and for the fish group overall.

2.7 | RT-qPCR

Gill samples from the fresh water and sea water were examined with RT-qPCR for *Candidatus* Branchiomonas cysticola and salmon gill poxvirus, whereas samples from sea water, in addition, were analysed for *Desmozoon lepeophtherii* and *Neoparamoeba perurans*. Nucleic acid extraction and RT-qPCR-analysis were performed as reported previously (Østevik et al., 2022).

Reverse Ct-values were calculated as follows:

$$\text{Reverse Ct} = 40 - \text{Ct pathogen}$$

Reverse Ct for negative samples was set to 0. Median reverse Ct-values and proportion of positive samples were used to assess the development of infection per fish group and sampling point.

FIGURE 2 Normal gill tissue and histology lesions recorded as counts and percent. All tissues stained with haematoxylin and eosin. (a) Almost normal gill tissue at low magnification, very few foci of pathology are seen. Bar 2 mm. (b) Normal gill tissue at high magnification. Bar 200 μm. (c) Multifocal vascular lesions and focal segmental hyperplasia, fish group B2, 3% of lamella with vascular lesions. Bar 4 mm. (d) Vascular lesions, high magnification. Aneurysms with associated lamellar epithelial hyperplasia and variable extent of recanalization (arrowhead). Bar 100 μm. (e) Multifocal segmental hyperplasia affecting both proximal and distal aspects of the filaments, fish group G2, 21% of gill tissue affected. Bar 3 mm. (f) Lamellar epithelial hyperplasia with amoeba (arrowheads), also note subepithelial inflammation (arrows) and haemorrhage, fish group G2, same fish as (e). Bar 100 μm. (g) Lamellar epithelial hyperplasia and inflammation of the distal aspects of the filaments – fish group A1, 33% of gill tissue affected. Bar 4 mm. (h) High magnification of inflammation shows loss of lamella (arrowheads) and expansion of the filament by fibrous tissue with mild inflammatory infiltrates (*) and hyperplasia and inflammation (arrow) in surrounding lamellar epithelium. Fish group A2. Bar 200 μm
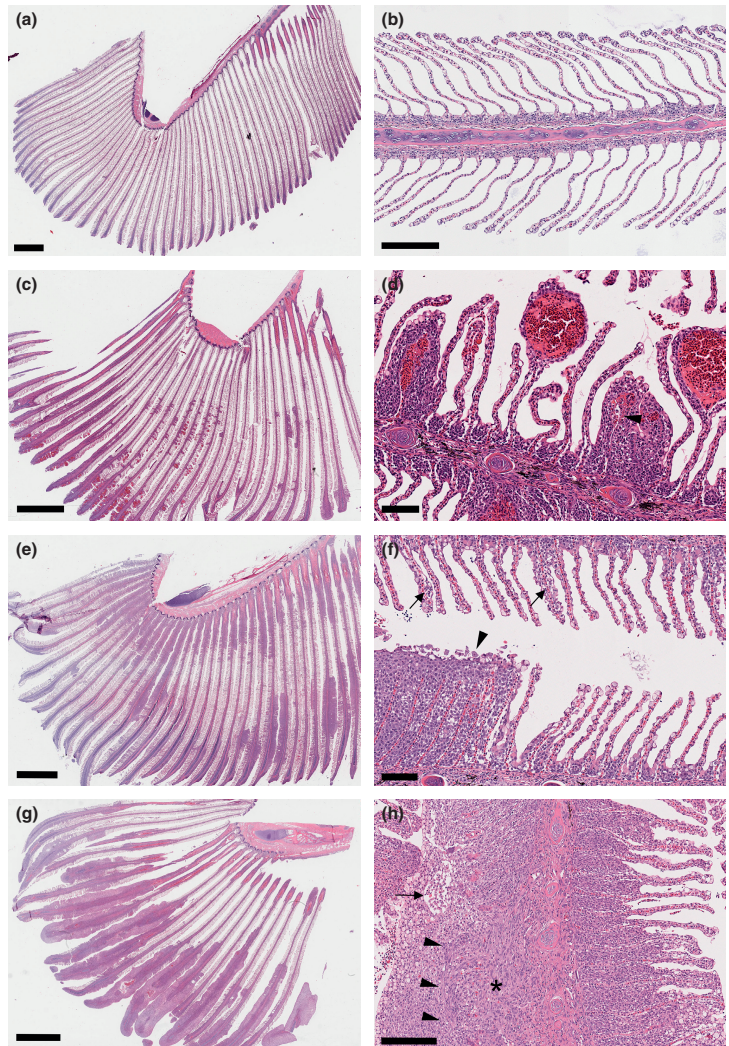
2.8 | Non-medicinal delousing

Information on the treatment method, start date and number of sea lice treatments per fish group was provided by the farming company.

2.9 | Plankton sampling and analysis

Sampling of sea water for classification and quantification of gelatinous zooplankton (jellyfish) and phytoplankton was conducted throughout the sea phase. In addition, farm staff was asked to record observations of jellyfish blooms occurring during the study period. A total of 29 to 56 water samples per site were examined for the presence of jellyfish and phytoplankton ($n = 323$ and $n = 322$). Plankton was collected using 250-μm mesh nets, with 25 cm and 50 cm diameter ring for phyto- and zooplankton, respectively. Two vertical net hauls (10 m depth) were collected and zoo- and phytoplankton samples were fixed with formalin or iodine, respectively, prior to examination at the laboratory in Oslo. Phytoplankton levels were quantified and classified by examination of a subsample in a Sedgewick counting chamber slide under an inverted microscope. The number of microscopic jellyfish was quantified and classified by examination of a subsample in a cell culture bottle under a stereomicroscope. For a detailed description of plankton sampling and assessment, see File S2.

2.10 | Descriptive statistical analysis

Statistical analysis was undertaken in STATA (StataCorp. 2015. Stata Statistical Software: Release 14. College Station, Texas, United States). Line plots were produced to provide a visual presentation of histopathology results, RT-qPCR results, gross scores, mortality data, water temperature, zoo- and phytoplankton levels and management procedures per fish group over time at sea. Trends for the development of gill infections, gill-related mortality, gross scores and gill histopathology over time and by season were described based on these graphs. Possible covariation and associations between the three main gill outcomes; gill-related mortality; gross scores and histopathology results, and between exposures pathogen prevalence and density and zoo-and phytoplankton levels were also assessed at fish group level based on these graphs.

Possible associations between pathogen load and detection by RT-qPCR and pathogen observation in tissue sections per fish group were assessed by producing scatter plots and computation of Spearman's rank-order correlation coefficient. To assess whether the extent of tissue lesions was increasing with increased pathogen load in the fish-level data set an ordinal variable was generated for each pathogen. 0 indicated not detected, 1 a low amount of genetic material (Ct-values were higher than 25), 2 a moderate amount of genetic material (Ct-values were between 20 and 25) and 3 a high amount of genetic material (Ct-values were lower than 20). Associations between pathogen load and gill histopathology or gill gross score were assessed by producing box and whisker plots of percent of tissue lesions or mean gross scores by pathogen loads 0 to 3, and by computation of Spearman's rank-order correlation coefficient for percent tissue lesions or mean gross score and reverse Ct-values for the different lesion and pathogen combinations. To determine if there was an association between epithelial cell necrosis and pathogen load, we used a series of logistic regression models with epithelial cell necrosis as the response variable and pathogen load (ordinal variables) as predictor variables. The odds ratio (OR) was calculated using the PCR-negative fish as a baseline.

3 | RESULTS

3.1 | Gill health in the freshwater phase

Histopathological lesions were absent or minimal to mild suggestive of overall good gill health in all the sampled fish groups ($n = 6$). One fish group (D) experienced SGPVD-related mortality (3.36% accumulated mortality), but the mortality had ceased when project samples were collected. SGPV was detected with RT-qPCR in four fish groups (C, D, G and H), from two freshwater facilities (sites 3 and 4). The prevalence of infection ranged from 0 to 53% across fish groups and time points. *Ca.* B. cysticola was only found in fish group E. No gill-related mortality, nor severe histopathological lesions or high gross gill scores were detected during the sea phase for fish groups

D1 and D2, the fish groups that experienced gill-related mortality due to SGPVD in the freshwater phase.

3.2 | Environmental data

Sea temperatures during the project period ranged from 4.2 to 17.7°C across all sites and time points and followed a clear seasonal pattern (Figure 3). The lowest mean temperatures were recorded in March (6.1°C, range: 4.4 to 8.6°C) and the highest in August (14.4°C, range: 10 to 16.9°C). The salinity at sea site B ranged from 27.6 to 30.9‰, without any evident seasonal pattern. At site C, salinity showed considerable variation throughout the year (range: 14.11 to 29.14‰) and was lowest in late summer and autumn (August to October). A single measurement of 20.62‰ was recorded for site H. The salinity at the remainder of the sites was unknown.

3.3 | Gill-related mortality

Presumed gill-related mortality was observed in 10 of 16 fish groups (Figures 3a and b), whereas no gill-related mortality was recorded at sites C, D and F. The mortality assigned as gill-related was generally low with the accumulated gill-related mortality ranging from 0.04 to 1.69% (Table S2). Gill-related mortality was observed both in the spring, summer, winter and autumn months. The highest gill-related mortality occurred at sites G and H. Mortality at site G coincided and followed a peak in severity and extent of histopathology lesions and a high prevalence and load of *N. perurans*. The gill-related mortality at site H occurred late in the production cycle when sampling and gross scoring were completed, thus the extent of gill pathology and prevalence and load of potential pathogens at the time were unknown.

3.4 | Gross gill pathology

Median gross gill scores across the project period were mostly below 1 (Table 2), but in five autumn-transferred fish groups (A1–2, B1–2 and C1), more than 50% of the scored fish had a mean gill score higher than 1 towards the end of the production cycle (Figures 3c and d). In groups A1, B1 and B2, the increase in gross pathology coincided with low levels of gill-related mortality. Fish group C1 developed bacterial branchitis and likely gross gill lesions as part of systemic bacterial infection, but mortality associated with this condition was recorded as infectious disease-related mortality. No increase in gross gill scores was evident during the time period when sea site G experienced gill-related mortality, the increased extent of gill histopathology and a high prevalence and load of *N. perurans*. Overall, gross gill scores did not appear to show a consistent seasonal variation or consistent covariation with water temperature, or prevalence of the putative gill pathogens detected by RT-qPCR analysis.
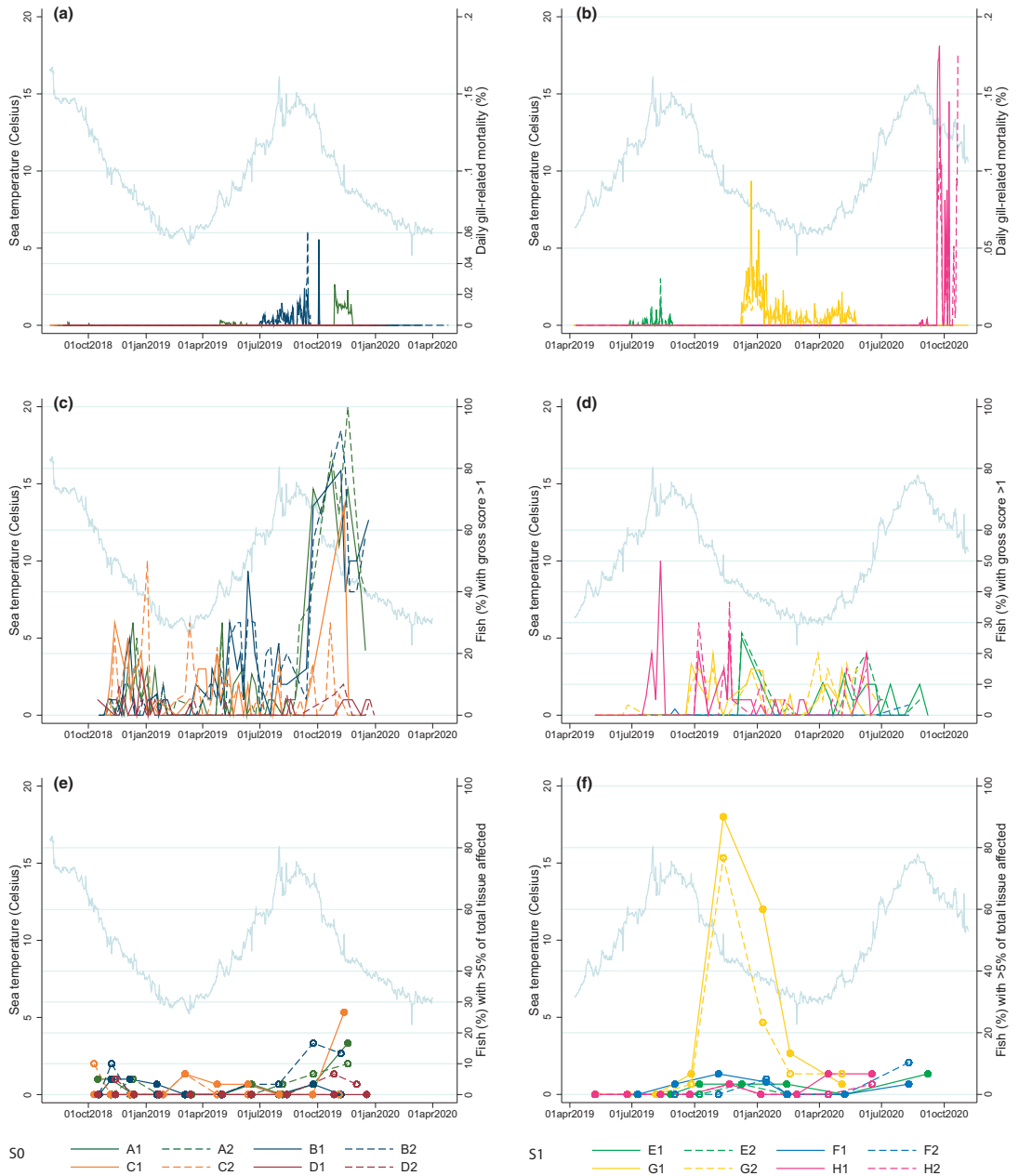
FIGURE 3 Sea temperature, daily gill-related mortality (%), percentage of fish with gross gill score >1 and percentage of fish with >5% of gill tissue affected (histopathology) over time at sea per fish group. Daily gill-related mortality (%) for (a) autumn-transferred fish and (b) spring-transferred fish, percentage of fish with gross gill score >1 for (c) autumn-transferred fish and (d) spring-transferred fish, percentage of fish with >5% of gill tissue affected (histopathology) for (e) autumn-transferred fish and (f) spring-transferred fish. The mean daily sea temperature across all study sites is shown in each figure

3.5 | Gill histopathology

The number and prevalence of fish with different lesions and pathogens observed and median, minimum and maximum lesion counts and percent across all samples are shown in Tables 3 and 4. The prevalence of lesions and pathogens per fish group is available in Table S2. There was a tendency towards a seasonal variation with more gill lesions observed in autumn and winter. The most severe gill lesions were also detected during or following periods of high sea temperatures in autumn and winter months (Figures 3e and f).

The histopathological lesions observed in the fish groups were generally mild, with the exceptions of fish groups G1 and G2 and to a lesser extent C1, A and B. The median total percent affected gill tissue was generally <2% across all time points and fish groups but ranged from 0 to 52% amongst individual fish. The exception to this was fish groups G1 and G2, where an increase in the median percent affected tissue to higher than 8% was observed during late autumn and winter of 2019. The pattern was similar for the proportion of fish with more than 5% of gill tissue affected. The first autumn and winter at sea more than 60% of fish had lesions affecting at least 5% of gill tissue at site G. In contrast, less than 10% of the sampled fish had this extent of gill lesions at any time in most of the remaining groups (Figures 3e and f).

The median percent lamellar epithelial hyperplasia and/or hyperplasia and inflammation generally showed a similar pattern as percent total tissue affected across fish groups and time points, though more vascular lesions than hyperplastic lesions were observed at some sites and time points. The highest median percent hyperplasia was found in groups G1 and G2, coinciding with the highest median total gill tissue affected. Median percent vascular lesions were less than 1% for all fish groups and time points, and the highest median percent vascular lesion was found in group G1 concurrently with the highest median percent tissue affected. In general, the median percent vascular lesions appeared to increase with increasing time at sea for autumn-transferred fish, but this pattern was not evident for spring-transferred fish. The extent of gill tissue with vascular and hyperplastic lesions in individual fish ranged from 0 to 30% and 0 to 52%, respectively. Overlap of hyperplastic and/or inflammatory lesions and vascular lesions were found in 34% of fish overall but did not constitute a substantial part of the histopathology observed in most fish groups.

Necrosis of lamellae was rarely observed (0.44% of gills sampled) and did not substantially contribute to the total percent affected gill tissue. Necrotic lamella was most often associated with bacterial infection and/or foreign material trapped between filaments

(Figures 4a-c). Epithelial cell necrosis or apoptosis was observed in a minority of fish in all fish groups and did not appear to have a clear seasonal distribution. Chronic inflammation of the filaments, deformed filaments and lamellar oedema were relatively rarely observed across the fish groups and time points (Figures 2g, h and 4d). No consistent increase in the proportion of fish with deformed filaments was observed over the time at sea. Chronic inflammation of the filaments was observed in more than 40% of fish from fish groups A1–A2 and B1–B2 during the second autumn at sea coinciding with an increase in hyperplastic and vascular lesions. An increase in the prevalence of lamellar oedema was seen in fish groups C1, F1 and F2 at different time points. The increase coincided and was associated with lesions of bacterial branchitis for fish group C1, but no specific disease diagnosis, changes in pathogen density or management operations were associated with the increase in fish groups F1 and F2.

3.6 | Gill pathogens

The prevalence of microorganisms observed in tissue sections across fish groups and per fish group is shown in Table 4 and Table S2. The prevalence, median reverse Ct-values and number of fish with mild, moderate and high pathogen load detected by RT-qPCR across all fish groups are available in Table 5. The median reverse Ct-values and prevalence per fish group over time are shown in Figure 5 and Figure S1, respectively.

SGPV was detected sporadically during the seawater phase with a prevalence ranging from 0 to 100% and seemed to have a seasonal distribution with positive samples collected in summer and autumn at most sites (Figure S1a and b). Overall SGPV was detected in 13.7% of the tested gills, the majority of which contained low amounts of viral genetic material (Table 5, Figures 5a and b). However, all fish groups tested positive for SGPV at least once at sea even if they were negative prior to sea transfer. Further, in several fish groups, SPGV appeared to disappear in late winter and early spring before being detected again during the following summer.

All fish groups became positive for *Ca.* B. cysticola and *D. lepeophtherii* after sea transfer and prevalence remained high (60–100%) throughout the sea phase (Figures S1c-f). Most positive gill samples contained low amounts of *D. lepeophtherii* genetic material, whereas close to half of the tested gills (47.6%) contained moderate to high amounts of *Ca.* B. cysticola genetic material (Table 5). A consistent seasonal variation of prevalence and pathogen load was not evident for *Ca.* B. cysticola or *D. lepeophtherii* (Figures 5 and

	Median	#fish ≥1	#fish >0
Gross pathology	**Min–Max**	**%fish ≥ 1**	**%fish > 0**
Gross score (mean all scored arches)	0	1053	7744
	0–5	6.77	49.79

TABLE 2 Summary of gross scores for all fish groups across the sea phase of the study (*n* = 15,553)

TABLE 3 Overview of histopathology lesions recorded as counts and percent for all fish groups across the sea phase of the study ($n = 3897$)

Lesion	Counts(median) Min−Max	% (median) Min−Max	#fish ≥5% %fish ≥5%	#fish > 0% %fish > 0%
Acute vascular lesions	2	0.02	1	2585
	0–833	0–5.68	0.03	66.33
Non-acute vascular lesions	12	0.09	21	3360
	0–5885	0–30.32	0.54	86.22
All vascular lesions	18	0.14	26	3617
	0–5885	0–30.32	0.67	92.81
Hyperplasia and hyperplasia and inflammation	14	0.1	120	3300
	0–5119	0–51.84	3.08	84.68
Overlap vascular lesions and hyperplasia	0	0	3	1330
	0–754	0–6.54	0.08	34.12
Total gill tissue affected	45	0.31	162	3836
	0–5988	0–51.90	4.16	98.43

S1). All autumn-transferred fish groups were positive for *D. lepeophtherii* at the first sample point 54 to 84 days after sea transfer. *D. lepeophtherii* were detected in most of the fish groups transferred in June 2019 at the second sample point at 74 to 84 days after sea transfer, whereas the parasite was not detected in fish groups transferred in April 2019 until the third or fourth sampling point at 124 to 172 days after sea transfer. For all fish groups, median reverse Ct-values peaked relatively rapidly after the first detection during the first autumn at sea. Most fish groups were negative for *Ca.* B. cysticola at the first sample point, but the bacterium was detected in all autumn-transferred fish groups by the second sample point at 84 to 96 days after sea transfer, whereas the first detection in most spring-transferred fish groups was at 120 to 138 days after sea transfer. After infection, median reverse Ct-values peaked rapidly during the first autumn at sea at some sites (C, G and H), whereas the peak occurred during the subsequent spring for the remaining sites.

Intraepithelial intracytoplasmic bacteria (epitheliocysts) were found in all fish groups after sea transfer, but prevalence varied markedly between fish groups and time points. A consistent seasonal variation was not observed. Epitheliocysts were observed in 50.7% of *Ca.* B. cysticola PCR-positive gills. The correlation between intracellular bacteria observed in the tissue sections recorded as proportion per fish group and time point and proportion of *Ca.* B. cysticola PCR-positive samples were strong (Spearman's rho =0.57, Prob > $|t|$ = 0.0000, n = 139). There was no clear association between increasing proportion of fish with epitheliocysts and increasing median reverse Ct-values for *Ca.* B. cysticola on the fish group level, and the variation in the proportion of fish with epitheliocysts at a similar median reverse Ct-level was large (Figure 7a). A consistent covariation between the proportion of *Ca.* B. cysticola PCR-positive fish and fish with intracellular bacteria in the lamellar epithelial cells was not observed for the different fish groups and time points.

Amoebic gill disease as diagnosed by the presence of amoeba and segmental lamellar epithelial hyperplasia (Figures 2e and f) was found in all but fish group C2. Amoeba without associated lamellar epithelial hyperplasia was not identified in any of the samples. *N. perurans* genetic material was detected in all fish groups, and the prevalence of both infection and AGD-diagnosis ranged from 0 to 100% across time points and fish groups (Tables 4 and 5). Presumed amoeba and histopathological lesions consistent with AGD were observed in 8.5% of gills and in 36.6% of *N. perurans* PCR-positive gills. There was a strong correlation between the proportion of gills with the observation of amoeba and the proportion of fish with PCR detection of *N. perurans* (Spearman's rho =0.90, Prob > $|t|$ = 0.0000, n = 139). For median reverse Ct-values higher than 10, there was an association between the proportion of fish with amoeba/ AGD observed in tissue sections and the median reverse Ct-value (Figure 7b). There was a consistent covariation between the proportion of *N. perurans* PCR-positive fish and fish with amoeba/AGD across fish groups and time points, though the proportion of fish with AGD/amoeba was considerably lower than the proportion of fish with positive PCR tests.

The presence and load of the parasite and the diagnosis of AGD by histopathology showed a seasonal distribution, being detected during late summer, autumn and winter and disappearing in spring and early summer (Figures 5g-h and 6). A histopathologic diagnosis of AGD or detection of the parasite by RT-qPCR was not necessarily coinciding with severe and extensive lamellar epithelial hyperplasia (Figure 6), or gill-related mortality or increased gross gill scores. However, markedly higher median reverse Ct-values in fish groups G1 and G2 (Figures 5g and h) coincided with a high prevalence of *N. perurans* infection and AGD diagnoses, the most severe histopathological lesions and gill-related mortality.

Excluding the fish with pasteurellosis from site C and intraepithelial intracytoplasmic bacteria (epitheliocysts), bacteria were sporadically

TABLE 4 Summary of histopathology lesions and pathogens recorded as dichotomous variables for all fish groups across the sea phase of the study ($n = 3897$)

Lesion/organism	# Fish	% Fish
Chronic inflammation filament	195	5.0
Deformed filaments	352	9.03
Lamellar oedema	185	4.75
Haemorrhage	2855	73.26
Necrosis whole lamella	17	0.44
Epithelial cell necrosis	341	8.75
Intracellular bacteria (Epitheliocysts)	1630	41.83
Trichodina spp.	41	1.05
Crustaceans	145	3.72
Filamentous bacteria	1	0.03
Other Bacteria	21	0.54
Metacercaria	42	1.08
Ichthyobodo spp.	28	0.72
Amoeba/AGD[a]	330	8.47
Foreign material[b]	18	0.46

[a]This category includes both the observation of parasites morphologically consistent with amoeba and AGD diagnosed based on the presence of these parasites and typical histopathological lesions.
[b]The presence of foreign material was only recorded when the material was associated with a tissue reaction as for instance inflammation or hyperplasia.

observed in association with or within gill tissue and only in 0.13% of the fish. Filamentous rod-shaped bacteria, most likely *Tenacibaculum* spp., were observed in just one fish that also had pasteurellosis and were associated with focal necrotizing branchitis (Figure 4c). Parasites *Trichodina* spp., *Ichthyobodo* spp., encysted metacercaria and small and large crustaceans were rarely found within or associated with the gill tissue (Figure 4e and f, Table 4). No clear seasonal distribution was evident, but the most fish with *Trichodina* spp. and *Ichthyobodo* spp. were found at site G when the most severe gill histopathology was detected.

3.7 | Association between gill lesions and pathogens

Segmental hyperplasia and unicellular parasites consistent with amoeba were the main histopathological findings in fish with moderate to marked gill pathology at site G during autumn and winter 2019 (Figure 2e and f). However, lamellar subepithelial inflammation and pale, yellow to brown, intracellular, granular pigment, and intracellular bacteria (epitheliocysts) were also observed in most fish. A high load and 100% prevalence of *N. perurans* coincided with the increase in gill pathology. A moderate to high load of pathogens *D. lepeophtherii* and *Ca.* B. cysticola was observed at the same time points, but similar loads of these pathogens were observed in other fish groups

and at other time points without the associated increase in gill pathology seen at site G.

In fish group C1, intravascular fine rod-shaped bacteria associated with variable extent of haemorrhage, thrombosis, inflammation, necrosis and hyperplasia were found in 50% of fish at the last sampling point in November 2019 (Figure 4a). Mild AGD lesions and amoeba were also observed. *Pasteurella* spp. infection was confirmed in the fish group by bacteriology the month prior. In November 2019, chronic inflammation of the distal part of the filaments with epithelial hyperplasia and variable extent of vascular lesions and loss of the overlying lamella was the dominating finding in fish groups A1 and A2 (Figure 2g and h). When comparing with other sampling points and fish groups, no increase or higher prevalence and load of gill pathogens as detected by RT-qPCR appeared to be associated with these lesions. For fish group B2, the most severe gill pathology was observed in September 2019, and lesions at this time point were dominated by segmental hyperplasia and amoeba and to a lesser extent vascular lesions and chronic inflammation of the distal filaments as described for fish groups A1 and A2.

Correlations between histopathology lesions recorded as percent and reverse Ct-values in the fish-level data set were not significant, very weak or weak (Spearman's rho < 0.3) for the majority of pathogen and lesion combinations. A moderate association was only found for hyperplasia and reverse Ct-values of *N. perurans* (Spearman's rho $= 0.34$, Prob $> |t| = 0.0000$, $n = 3885$). Correlations between mean gross scores and reverse Ct-values in the fish-level data set were also not significant, very weak or weak (Spearman's rho < 0.3) for all pathogens. Examination of box and whisker plots of percent of tissue lesions in gills with no, mild, moderate or high pathogen load (ordinal RT-qPCR results) showed an increasing extent of hyperplasia with an increasing amount of *N. perurans* genetic material detected (Figure 8). Similarly, there was an increasing extent of total tissue affected with an increasing amount of *N. perurans*, whereas there was no clear or only a very mild tendency of increasing extent of hyperplasia, total tissue affected, vascular lesions or overlap of vascular and hyperplastic lesions with increasing pathogen load for the remainder of the pathogens. Correlation between reverse Ct-values for the different pathogens was not significant, very weak or weak (Spearman's rho < 0.3), except for between *N. perurans* and *D. lepeophtherii* (Spearman's rho $= 0.34$, Prob $> |t| = 0.0000$, $n = 3933$). There was an increasing number of fish with epithelial cell necrosis with an increasing load of SPGV, *N. perurans* and *Ca.* B. cysticola (Table 6), but the association was strongest for SGPV. The probability (odds) of observing epithelial necrosis in the gills of fish with a moderate load of SGPV was 21.60 times higher compared to fish testing negative for SGPV. The correlation between the mean gross score and total tissue affected as assessed by histopathology was weak (Spearman's rho $= 0.24$, Prob $> |t| = 0.0000$, $n = 3541$). Similarly, the correlation between the total tissue affected and the gross score of the arch that was sampled for histopathology (2nd left arch) was also weak (Spearman's rho $= 0.25$, Prob $> |t| = 0.0000$, $n = 3593$).

FIGURE 4 Lesions and pathogens observed. (a) Bacteria, haemorrhage, necrosis and lamellar epithelial hyperplasia. Note basophilic granular material (bacteria) partially embedded in eosinophilic material (fibrin) expanding filament vessels (*) and lamellar sinusoids (arrowheads). *Pasteurella* spp.– infection in fish group C1. Bar 300 μm. (b) Foreign material, possibly plant material (*), caught between filaments. Inflammation and haemorrhage are seen in the filament and there are necrosis (loss) of surrounding lamella (arrowheads). Fish group F2. Bar 300 μm. (c) Necrosis of lamella with loss of normal tissue structures and large amounts of filamentous bacteria, likely *Tenacibaculum* sp., in the necrotic tissue. Inflammatory cells and haemorrhage are seen in the filament. Fish group C1. Bar 60 μm. (d) Focal proliferation of filament cartilage—possibly callus formation caused by previous trauma. Recorded as a deformity. Bar 400 μm. (e) Small crustacean between lamellae. Fish group A1. Bar 80 μm. (f) Metacercaria surrounded by a thin fibrous capsule in the filament. Fish group A2. Bar 50 μm
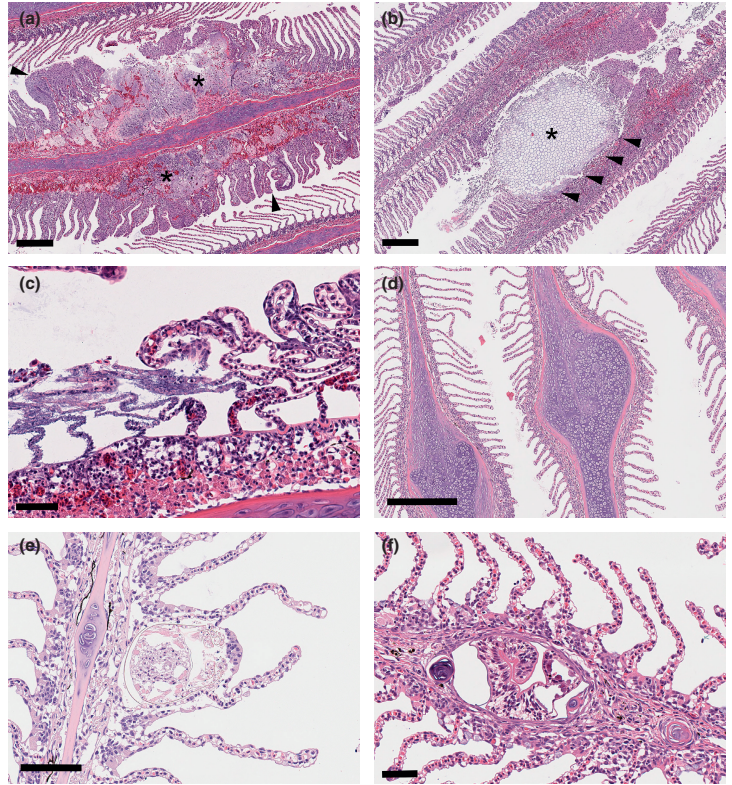
TABLE 5 Summary of pathogens detected by RT-qPCR for all pens across the sea phase of the study (*n* = 3933). Numbers in the column headings indicate low, moderate and high pathogen load. 1 indicates Ct-values higher than 25, 2 indicates Ct-values between 25 and 20, 3 indicates Ct-values lower than 20

Organism	Median reverse Ct Min–max	Detection # fish positive %fish positive	Pathogen load ≥1 #fish %fish	Pathogen load ≥2 #fish %fish	Pathogen load ≥3 #fish %fish
N. perurans	0 / 0–26.49	772 / 19.63	388 / 9.87	291 / 7.40	93 / 2.36
Ca. B. cysticola	14.72 / 0–25.27	3083 / 78.39	1211 / 30.79	1459 / 37.10	413 / 10.50
D. lepeophtherii	10.69 / 0–23.27	3227 / 82.05	3076 / 78.21	146 / 3.71	5 / 0.13
SGPV	0 / 0–21.67	538 / 13.68	514 / 13.68	23 / 0.58	1 / 0.03

3.8 | Non-medicinal delousing

The type and number of sea lice treatments (excluding in-feed treatment) per fish group are summarized in Table S2. The number of delousing operations during the sea phase ranged from 0 to 11 between fish groups, with a median of five treatments per group.

Freshwater and hydrogen peroxide bath treatments that may impact the prevalence and median Ct-values of *N. perurans*, the prevalence of AGD and development of hyperplastic gill lesions, were performed in 13 fish groups (Table S2). A reduction in the proportion of *N. perurans* and AGD positive fish was seen at the sampling points immediately after one or more freshwater or hydrogen peroxide
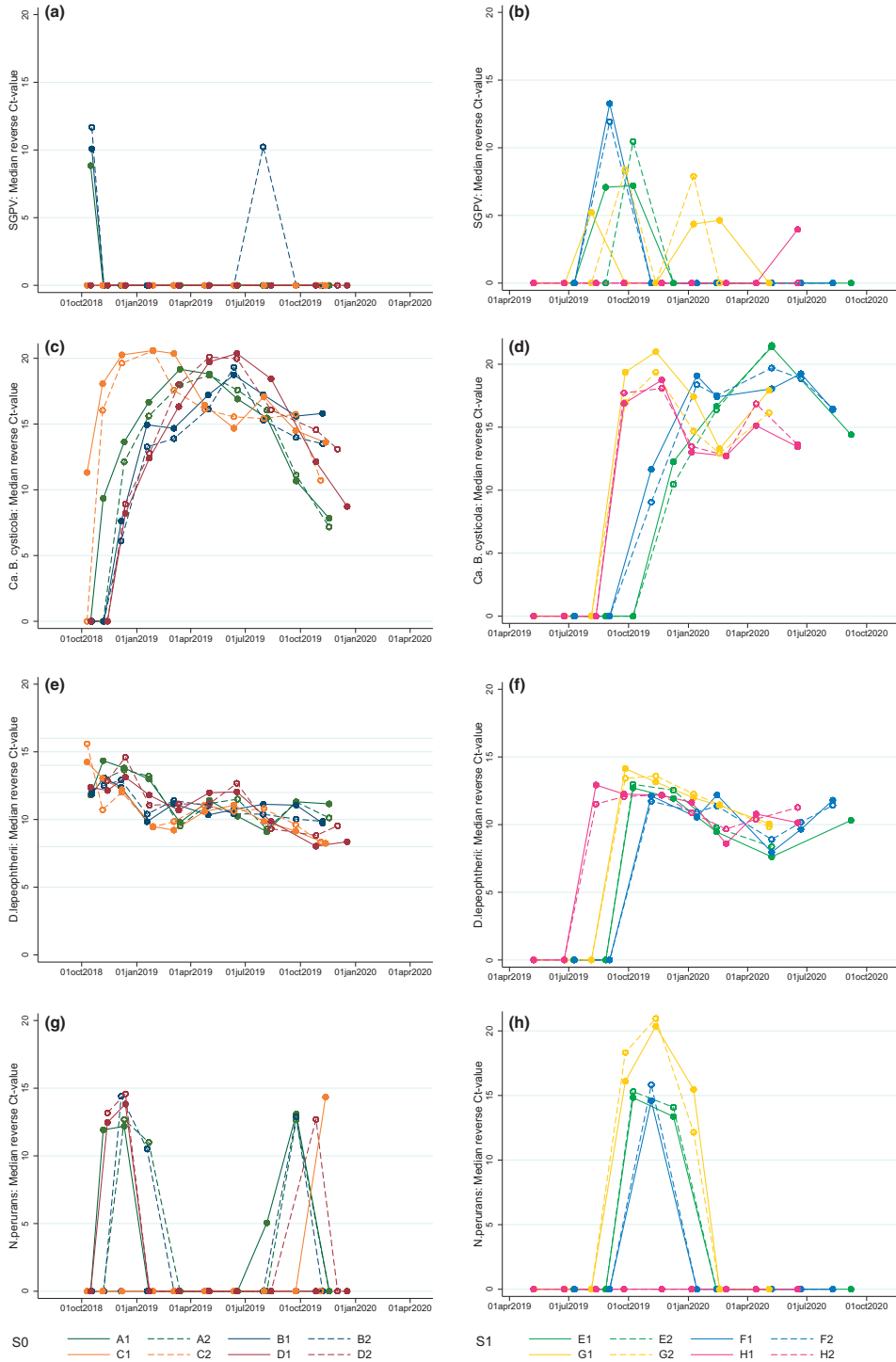
FIGURE 5 Median reverse Ct-values per fish group throughout the sea phase for autumn-transferred (S0) and spring-transferred (S1) fish groups. (a) SGPV (S0), (b) SGPV (S1), (c) *Ca.* B. cysticola (S0), (d) *Ca.* B. cysticola (S1), (e) *D. lepeophtherii* (S0), (f) *D. lepeophtherii* (S1), (g) *N. perurans* (S0), (h) *N. perurans* (S1)
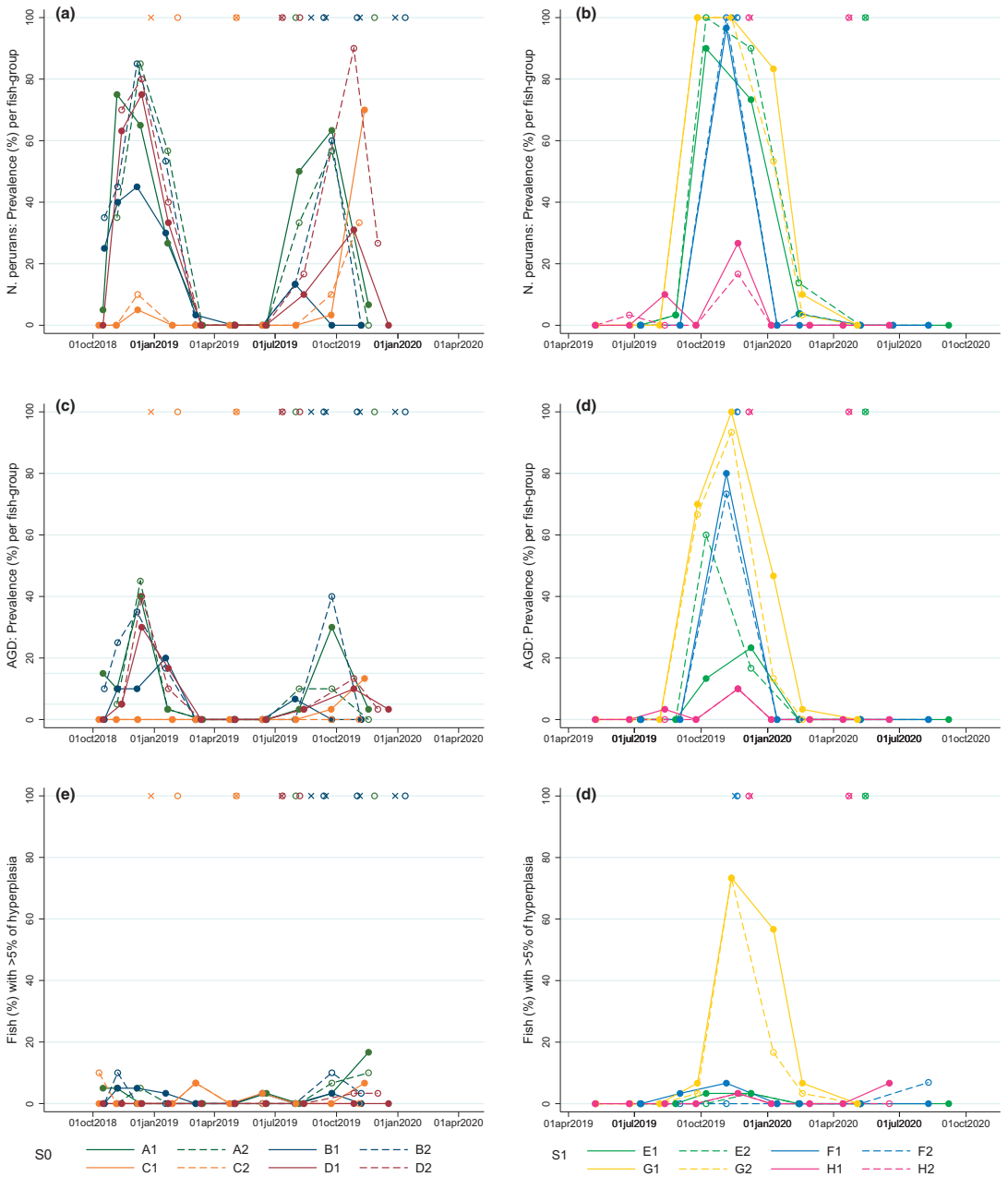
FIGURE 6 Prevalence of *N. perurans*, AGD and percentage of fish with >5% of gill tissue with lamellar epithelial hyperplasia and hyperplasia and inflammation over time at sea per fish group. (a) *N. perurans* (S0), (b) *N. perurans* (S1), (c) AGD (S0), (d) AGD (S1), (e) hyperplasia (S0), (f) hyperplasia (S1). Non-medicinal and medicinal sea lice treatment events that could impact the development of AGD and *N. perurans* prevalence are represented as x for fish groups A1-H1 and o for fish groups A2-H2
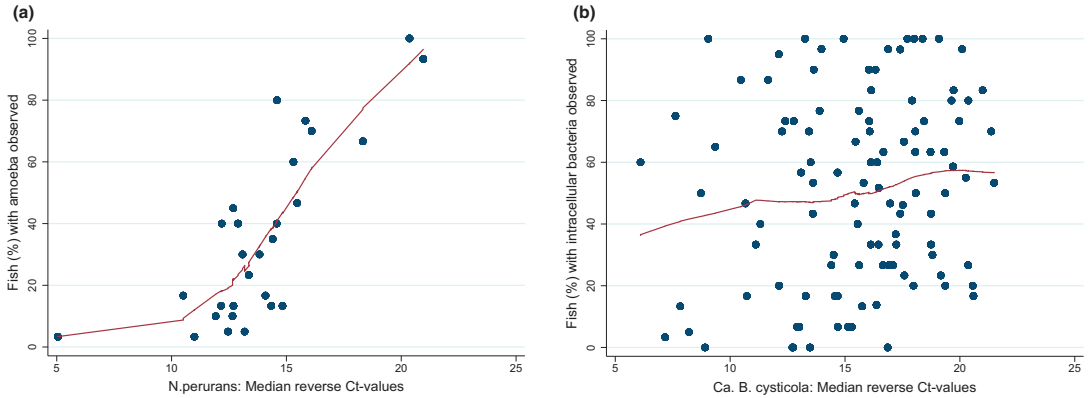
FIGURE 7 Relationship between pathogens observed in tissue sections and Ct-levels detected by RT-qPCR per fish group. Scatterplots show the percentage of fish per sampling point and fish group ($n = 139$) where a) amoeba and b) intracellular bacteria (epitheliocysts) were observed in the tissue sections against median reverse Ct-values for *N. perurans* and *Ca.*B. cysticola, respectively. Fish groups with a median reverse Ct of 0 are excluded for clarity leaving (a) $n = 28$ and (b) $n = 107$ fish groups. [Correction added on 12 July 2022, after first online publication: figure 7(a) has been updated in this version]
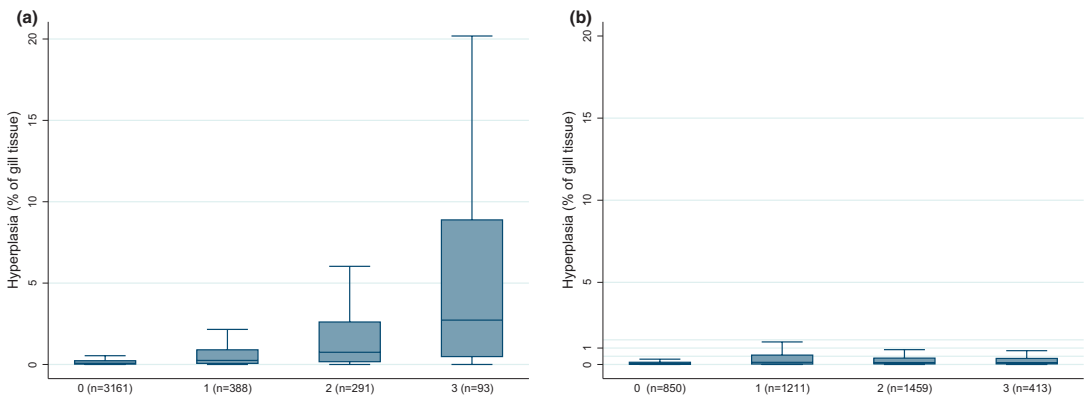
FIGURE 8 The extent of lamellar epithelial hyperplasia at different loads of pathogens detected in the gill tissue. Box and whisker plots show percent lesions ($n = 3885$) grouped by ordinal RT-qPCR-results for (a) *N. perurans*, (b) *Ca.* B. cysticola. Outliers are excluded for clarity

bath treatments in six fish groups, whereas an increase was seen in five groups (Figure 6).

3.9 | Jellyfish and phytoplankton

The levels of plankton were generally low with the highest levels of phytoplankton detected during spring and summer at sites C and D (data not shown). The number of microscopic jellyfish was < 50 organisms/m³ with the highest number during the spring months. Based on the submitted samples, there were no apparent phytoplankton or jellyfish blooms during the study period. Furthermore, observation of jellyfish blooms was not reported from any of the sea sites. No increase in gross gill scores, gill histopathology, gill-related

mortality or total mortality was evident following peaks in total phytoplankton levels to 732,164 and 535,779 cells/L detected at sites C and D, respectively. Additionally, there was no covariation between phytoplankton levels and any of our gill indicators.

4 | DISCUSSION

Neoparamoeba perurans appeared to be the most important cause of gill pathology and specifically lamellar epithelial hyperplasia in this study, whereas no consistent covariation and no or weak associations between extent of hyperplasia and prevalence and load of SGPV, *Ca.* B. cysticola and *D. lepeophtherii* were observed. There were no or weak associations between vascular lesions and overlap of vascular

TABLE 6 Epithelial cell necrosis or apoptosis. Results of statistical analysis of the association between epithelial cell necrosis or apoptosis and pathogen load

Pathogen	Pathogen load	Odds ratio	$P > |z|$	95% Conf. Interval
N. perurans	0			
	1	1.21	0.331	0.82, 1.77
	2	2.62	0.000	1.88, 3.67
	3	7.82	0.000	5.03, 12.16
Ca. B. cysticola	0			
	1	1.02	0.934	0.71, 1.45
	2	1.60	0.005	1.16, 2.22
	3	3.02	0.000	2.06, 4.41
D. lepeophtherii	0			
	1	0.28	0.000	0.15, 0.52
	2	1.27	0.494	0.65, 2.48
SGPV	0			
	1	2.64	0.000	2.02, 3.46
	2	21.60	0.000	9.35, 49.86

and hyperplastic lesions and pathogen load for any of these pathogens and *N. perurans*. AGD appeared to resolve without treatment with falling sea temperatures in fish groups with mild infections and pathology. *D. lepeophtherii* and *Ca*. B. cysticola infections were established and persisted after sea transfer in all fish groups and a clear seasonal variation in pathogen load and prevalence were not observed. *Ca*. B. cysticola prevalence and prevalence of intracellular bacteria observation were highly correlated, but there was no association between the prevalence of intracellular bacteria observed and increasing pathogen load for *Ca*. B. cysticola. SGPV infection and prevalence showed a seasonal pattern varying with sea temperature. Fish groups negative for SGPV at sea transfer became infected, apparently cleared the infection before again testing positive which may indicate that previous SGPV infection does not protect against reinfection of the same fish group. Coinfection with three or more putative gill pathogens was found in all fish groups and appears to be the norm in sea-farmed Atlantic salmon in Western Norway.

4.1 | The impact of gill pathogens on gill health

Neoparamoeba perurans is an important cause of gill disease and gill pathology in salmonid aquaculture, and this has been firmly established since amoebic gill disease was first described in Tasmania (Munday, 1986; Young et al., 2007). Thus, the finding that high loads of *N. perurans* were associated and coincided with hyperplastic gill lesions and gill-related mortality was unsurprising. Our findings are also in agreement with two longitudinal studies from marine farms in Ireland and Scotland in which increasing *N. perurans* loads was associated with increasing gill histology scores (Downes et al., 2018; Herrero-Fernández, 2019). *N. perurans* load and prevalence of

amoeba and AGD pathology in tissue sections at group level were highly correlated, indicating an increasing prevalence of amoeba and AGD lesions with increasing pathogen load. However, as tissue sampling was standardized, different gill arches were collected for histopathology and RT-qPCR, and AGD was only diagnosed when amoeba was observed in the tissue section, the true prevalence of *N. perurans* and AGD in our fish groups was likely higher than reported here, and an even stronger association between RT-qPCR and histology would be expected if the same arch was sampled for these analyses (Adams, Ellard, & Nowak, 2004; Fringuelli, Gordon, Rodger, Welsh, & Graham, 2012).

As in the current study, increasing *Ca*. B. cysticola loads was not associated with increased histological gill scores or a clinical diagnosis of gill disease in recent longitudinal studies (Downes et al., 2018; Gunnarsson et al., 2017; Herrero-Fernández, 2019). These findings are further supported by the lack of clinical disease in fish infected with *Ca*. B. cysticola, SGPV and *Ca*. Piscichlamydia salmonis in a cohabitation study (Wiik-Nielsen et al., 2017). In contrast to the current study, an association between the amount and prevalence of epitheliocysts observed in the gill tissue and *Ca*. B. cysticola load has been reported previously. Further, an association between gill histopathology and high numbers of epitheliocysts was described (Mitchell et al., 2013; Steinum et al., 2010). *Ca*. B. cysticola is the predominant epitheliocyst-forming bacteria in gills of Atlantic salmon in Norway and Ireland, but other epitheliocyst-forming bacterial species exist (Mitchell et al., 2013; Toenshoff et al., 2012). Thus, the lack of association between the proportion of fish with epitheliocysts and load of *Ca*. B. cysticola in our material could indicate that some of the observed epitheliocysts contain other bacteria like *Candidatus* Piscichlamydia salmonis, *Candidatus* Syngnamydia salmonis or a new epitheliocyst-forming bacterium recently described (Wiik-Nielsen et al., 2015). However, *Ca*. B. cysticola can be present in gill tissues without forming intracellular cysts, so the lack of association may also be related to higher levels of bacterial colonization without epitheliocyst formation in the fish groups (Gjessing et al., 2021).

The relatively low prevalence and load of SGPV and the lack of association between SGPV load and the extent of proliferative lesions and overall gill pathology are in agreement with previous studies (Downes et al., 2018; Gjessing et al., 2019; Herrero-Fernández, 2019). The increased likelihood of observation of epithelial cell necrosis or apoptosis in fish with higher loads of SGPV is in line with studies demonstrating SGPV in the apoptotic gill epithelium of sea-farmed salmon with complex gill pathology (Gjessing et al., 2017; Gjessing et al., 2021). Whilst SGPV initially was suggested to be a primary pathogen that could pave the way for other gill pathogens (Gjessing et al., 2017), recent studies have shown that immunosuppression may be necessary for disease development in SGPV-infected fish (Amundsen et al., 2021; Thoen et al., 2020). The results of the longitudinal studies conducted so far suggest that SGPV is not an important cause of severe gill disease in sea-farmed Atlantic salmon, at least not in the populations examined, and the significance of SPGV infection and pathology in the sea water phase needs to be further explored (Downes et al., 2018; Herrero-Fernández, 2019).

We did not find a moderate to strong association or consistent covariation between the extent of gill pathology, gross gill score or gill-related mortality and _D. lepeophtherii_ loads in the current study. This is in contrast with previous studies reporting that higher _D. lepeophtherii_ loads were associated with gill disease, proliferative gill lesions, proliferative gill inflammation (PGI) and increased histology gill scores (Gunnarsson et al., 2017; Hamadi, 2011; Herrero-Fernández, 2019; Nylund et al., 2011; Nylund, Nylund, Watanabe, Arnesen, & Karlsbakk, 2010; Steinum et al., 2010). A possible reason for the discrepancy may be related to the low and moderate loads of _D. lepeophtherii_ and relatively few fish and fish groups with severe gill pathology in the current study. Histologic lesions reported to be associated with _D. lepeophtherii_ and/or _Ca._ B. cysticola, that is, ballooning degenerative cells containing pigmented material, lamellar epithelial hyperplasia, necrosis in hyperplastic lesions, pustules, subepithelial inflammation and necrosis of subepithelial cells were observed in several of our fish groups (Gjessing et al., 2019; Gjessing et al., 2021; Matthews, Richards, Shinn, & Cox, 2013; Weli et al., 2017). But, because the primary aim of the current study was to determine which factors might impact the overall gill health, we chose to provide an accurate estimate of the extent of gill tissue with the presumed most important and commonly observed gill lesions. Thus, the extent of lesions reported to be associated with high loads of _D. lepeophtherii_ and _Ca._ B. cysticola was not consistently recorded or quantified, and this could be pursued in future studies. However, the repeated finding of widespread _D. lepeophtherii_- and _Ca._ B. cysticola infection and persistence in healthy Atlantic salmon strongly suggest that predisposing or additional factors are necessary for these agents to cause gill lesions and gill disease and/or that there might be variation in virulence and pathogenicity within these species.

This study further demonstrates that coinfections of multiple putative gill pathogens are the norm in sea-farmed Atlantic salmon in Western Norway. Infection with three or more microorganisms was found in >20% of the fish, but the majority of fish groups did not develop severe gill pathology or associated mortality. As all fish groups were infected with all pathogens, it was not possible to determine if fish groups infected with a given pathogen had worse outcomes or higher pathogen loads after infection with a second or third pathogen. The prevalence and pathogen load of the four pathogens largely followed different patterns and there was only moderate correlation between pathogen load for _D. lepeophtherii_ and _N. perurans_. This might suggest an association in which infection with one pathogen allows and promotes the proliferation of another. Such a relationship might explain the frequent observation of gill lesions ascribed to different pathogens in the same fish. Another possibility is that higher sea temperatures during autumn favours the proliferation of these pathogens independent of infection status with the other.

A strong association or covariation between the extent of vascular lesions and pathogen load, or between concurrent vascular and hyperplastic lesions and pathogen load was not observed in our material. Whilst vascular lesions frequently were associated with hyperplasia of the immediately overlying and surrounding lamellar epithelium, aneurysms and thrombi were often not associated with severe inflammation or severe lamellar epithelial hyperplasia. Further, the chronic inflammation, lamellar epithelial hyperplasia and vascular lesions observed in fish groups A1 and A2 were not associated with higher loads of pathogens compared to other fish groups. These observations suggest that factors other than the gill pathogens included in this study could be involved in the development of these lesions. Vascular lesions are unspecific responses and are not characteristic of a particular insult. An increase in prevalence and severity of thrombi, haemorrhage and/ or aneurysms have been reported after non-medicinal delousing and _in situ_ net washing, and haemorrhage, thrombi and aneurysms have been observed after exposure to jellyfish (Baxter, Sturt, et al., 2011; Baxter, Rodger, McAllen, & Doyle, 2011; Bloecher et al., 2018; Marcos-Lopez, Mitchell, & Rodger, 2016; Mitchell, Baxter, & Rodger, 2011; Østevik et al., 2021; Østevik et al., 2022; Powell, Atland, & Dale, 2018).

Notably, extensive lamellar thrombosis and fibrinohemorrhagic and necrotizing branchitis were observed in fish with systemic bacterial infection (pasteurellosis). Pasteurellosis caused by the currently unofficially named _Pasteurella atlantica_ genomvar _salmonicida_ has become endemic in sea-farmed salmon in Southwestern and Western Norway since 2018. The disease manifests as a systemic bacterial infection with fibrinous polyserositis, necrosis and inflammation of internal organs, muscle abscesses, exophthalmia and ophthalmitis (Gulla, Nilsen, Olsen, & Colquhoun, 2020; Legård & Strøm, 2020). In the authors experience intravascular bacteria and histological lesions are frequently identified in the gills of fish with pasteurellosis. Disturbances of coagulation and haemostasis are also common in fish with other systemic bacterial infections (Salte, Nafstad, & Asgård, 1987). Thus, pasteurellosis has become a cause of gill lesions in Atlantic salmon in Western Norway, and it is possible that lamellar thrombi and vascular lesions are related to concurrent and/or previous systemic bacterial infection in some cases, even when bacteria are not observed in the gill tissue.

4.2 | The impact of season and sea temperature on gill pathogens

Neoparamoeba perurans infection in our fish groups showed a seasonal variation with the highest prevalence and pathogen load in autumn and winter, and clearing of infection in spring. This is consistent with the pattern observed in Norway since AGD first became endemic and is likely related to the seasonal variation in sea temperatures (Clark & Nowak, 1999; Mo et al., 2015; Sommerset et al., 2021). Our results also further demonstrate that _N. perurans_ infection and AGD can be self-limiting and resolve without treatment when sea temperatures fall (Clark & Nowak, 1999). Sites C and H with the lowest recorded salinities had the lowest overall prevalence of _N. perurans_ infection and AGD. Unfortunately, information about salinity at most sites and variation in salinity at site H over time was not available, but the low salinity likely explains the markedly

lower prevalence of AGD at sites C and H (Clark & Nowak, 1999; Mo et al., 2015; Oldham, Rodger, & Nowak, 2016).

In agreement with previous studies, there was a very high prevalence of *D. lepeophtherii* and *Ca.* B. cysticola infection in Southern and Western Norway, and infection was established after sea transfer and persisted amongst all fish groups (Gunnarsson et al., 2017; S. Nylund et al., 2011; Steinum et al., 2015; Steinum et al., 2010; Sveen, Overland, Karlsbakk, & Nylund, 2012). As reported by Sveen et al. (2012) we also found that *D. lepeophtherii* infection and peak *D. lepeophtherii* load occurred more rapidly after sea transfer for autumn-transferred fish compared to spring-transferred fish. This difference is likely related to the higher sea temperatures in autumn compared to spring. However, we did not observe a seasonal and temperature-dependent variation in pathogen load once fish groups were infected with these two pathogens. Rather in some fish groups, the highest levels of *Ca.* B. cysticola were found during the coldest periods of the year. The highest pathogen load of *D. lepeophtherii* was found during the first autumn at sea relatively early after fish groups became infected, but no consistent increase was seen in the subsequent autumn. This could suggest that the parasite may infect or proliferate at higher levels in recently sea-transferred naïve fish. SGPV infection at sea appeared to have a seasonal distribution with most infected fish detected in late summer and fall. The detection of SGPV infection after sea transfer in previously negative fish groups is consistent with previous findings that the virus spreads horizontally and suggests that SGPV infection also occurs in sea water (Gjessing et al., 2017; Wiik-Nielsen et al., 2017). The disappearance and reappearance of the virus throughout the sea phase suggest that fish groups may clear the virus and become re-infected multiple times over a production cycle. This contrasts with the lack of reinfection reported by Gjessing et al. (2018) and indicates that previous infection does not protect against reinfection of the same fish group. Alternatively, the virus may persist in the population at a very low prevalence and may proliferate and spread when conditions are more favourable.

4.3 | The impact of the site on gill infections, gill pathology and gill-related mortality

The patterns of gill infections, gill pathology and gill-related mortality within each farm site were markedly more similar than between sites indicating that factors relating to the site are important for these outcomes. This finding is perhaps not surprising as local environmental conditions and infection pressure likely have a considerable impact on gill health and gill infections. Fish groups at the same farm experience a similar environment and are generally exposed to the same type and number of management operations like net cleaning and delousing treatments. These fish also originate from the same stock and hatchery and had experienced similar environmental conditions and exposure to infectious agents before sea transfer. Further, staff performing gross gill scoring and cause-specific mortality classification may also contribute to

differences between sites because the local staff at each farm performed these tasks.

4.4 | Lacking impact of plankton levels on gill health

In the current study, there was no discernible impact of zoo- and phytoplankton on any of the gill outcomes or the total mortality. However, mostly low concentrations of plankton and jellyfish were detected, and *Pseudo-nitzschia* spp. and other diatoms were the dominating type of phytoplankton observed. *Pseudo-nitzschia* species can produce toxins (Amnesic Shellfish Toxins (AST), domoic acid (DA)) that can accumulate in the marine food chain and in mussels. The toxins can cause serious disease in humans consuming shellfish but are not reported to be associated with fish mortality or disease in Norwegian waters (Karlson et al., 2021). Whilst brief and transient phytoplankton and zooplankton blooms might have been missed because of our sampling regime, the lack of association between plankton levels and gill pathology and mortality in the current study is likely related to the lack of a substantial plankton bloom at any of the sites during the study period.

4.5 | Gill-related mortality and gill gross score as indicators of gill health

Gill-related mortality, gross gill scores and extent of tissue damage detected by histopathology were used as indictors of gill health status in this study. We found overall weak correlations between gross scores and the extent of microscopic gill lesions, and low correlations and little covariation between pathogen load and prevalence and gross scores. Our results contrast with several studies reporting good agreement between gross scores and histopathological scores, and gross scores and pathogen load in fish with moderate to severe AGD and *N. perurans* infections (Adams et al., 2004; Bridle, Crosbie, Cadoret, & Nowak, 2010; Clark & Nowak, 1999). However, Krol et al. (2020) examined the gill arch with the most gross lesions and found no difference in gill histopathology or gene expression in fish receiving different proliferative gill disease (PGD) gross scores, and questioned the usefulness of the PGD gross scoring system for diagnosis and monitoring of gill disease. Mild, focal lesions and diffuse inflammation or hyperplasia can be difficult or impossible to detect grossly (Clark & Nowak, 1999). Thus, the lack of severe pathology and high pathogen load in most of the sampled fish likely contributed to poorer correlation between gross and histopathological assessments in the current study (Adams et al., 2004; Clark & Nowak, 1999; Collins et al., 2017). The area of the gill examined by gross scorers and the histopathologist overlapped but differed which is likely to further reduce agreement (Adams et al., 2004; Taylor, Wynne, Kube, & Elliott, 2007). Lastly, the inclusion of multiple gross scorers with variable experience and the variation of

scorers over time, across and within sites, in addition to the use of a scoring system that was new to the scorers, likely introduced variation into the gross score observations unrelated to actual variation in gross pathology (Adams et al., 2004). In conclusion, the overall poor correlation between gross and microscopic lesions is likely explained a combination of factors, from gross scorers, scoring system, histology sampling protocol and the inability to detect mild lesion grossly. However, despite moderate to severe microscopic gill lesions and a high load and prevalence of *N. perurans* at site G, a concurrent increase in gross score was not recorded for all left arches overall or for the second left gill arch sampled for histology. This observation indicates that cases of moderate to severe gill pathology and AGD may go undetected if gills are only assessed by gross examination.

Cause-specific mortality was assigned as gill-related based on gross examination of dead fish by site staff and/or fish health personnel, information from diagnostic reports and knowledge about environmental and managerial events at the site. Due to the assignment of a single cause and lack of gross examination of each dead fish, there is a risk of underestimating the contribution of infectious diseases to the mortality observed at a site. From human studies of verbal autopsies, it has been shown that it is easier to correctly classify mortality caused by acute, severe trauma, than infectious or cardiovascular disease (Lozano et al., 2011). Commonly, all mortalities occurring after a handling or treatment event at a site are recorded as treatment-related mortality irrespective of other diseases present in the population or gross findings suggestive of concurrent infectious disease. This may lead to further overestimation of observable management events as causes of mortality and an underestimation of the contribution of infectious and non-infectious disease to the observed mortality. Individual fish dying of diseases in which gross lesions are not readily apparent or non-specific are also more likely to be incorrectly classified. However, despite the weaknesses, cause-specific mortality classification has the advantage of being fast, cheap, can be performed on-site and by site staff and consider data from concurrent laboratory analysis. The resulting data provide an estimate of the drivers of mortality at the population level and can be used for future statistical analysis. But these data were not intended or suited for studies of the interactions between pathogens or interactions between infectious, managerial and environmental factors causing gill disease. Further, the gill-related mortality data should be considered a very conservative estimate of (severe) gill disease in a population.

4.6 | Limitations of the study

In addition to the limitations related to the standardized sampling and histopathological assessment discussed previously, there are some limitations of this study related to the fact that this was a field study performed at sites in commercial production. During the study period at sea several fish groups were split or mixed with other fish groups. Mixing leads to a dilution of the study fish group with fish from one or more other pens that may have another disease history. In contrast, the splitting of a fish group is not problematic if groups were randomly split, but unfortunately, whether specific criteria were applied during the splitting of one or more of our fish groups was unknown. The inability to follow the same cohorts throughout the production cycle could have impacted results for the sampling points following the mixing and splitting events and lead to an uncertainty in the estimate of the prevalence and extent of gill lesions and gill infections within a fish group over time. The information obtained from subsets of sampled fish were presumed to be representative of their respective fish groups. To ensure representative samples, randomized sampling is ideal yet impossible to achieve when performing field sampling at commercial sea farms. The use of food to lure fish towards the surface during sampling may have led to the collection of fish with better health than the general population because diseased fish can become anorexic and lethargic. To have a greater chance of detecting gill disease within the population, up to 15 fish with clinical signs of disease should have been sampled if available. Unfortunately, for a considerable proportion of the samples, fish health status was not recorded and it is unknown if these fish displayed clinical signs of disease. Nonetheless, it is possible that the sampling method led to an underestimation of the degree of gill pathology and gill infections in the fish groups.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

Research data are not available.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.
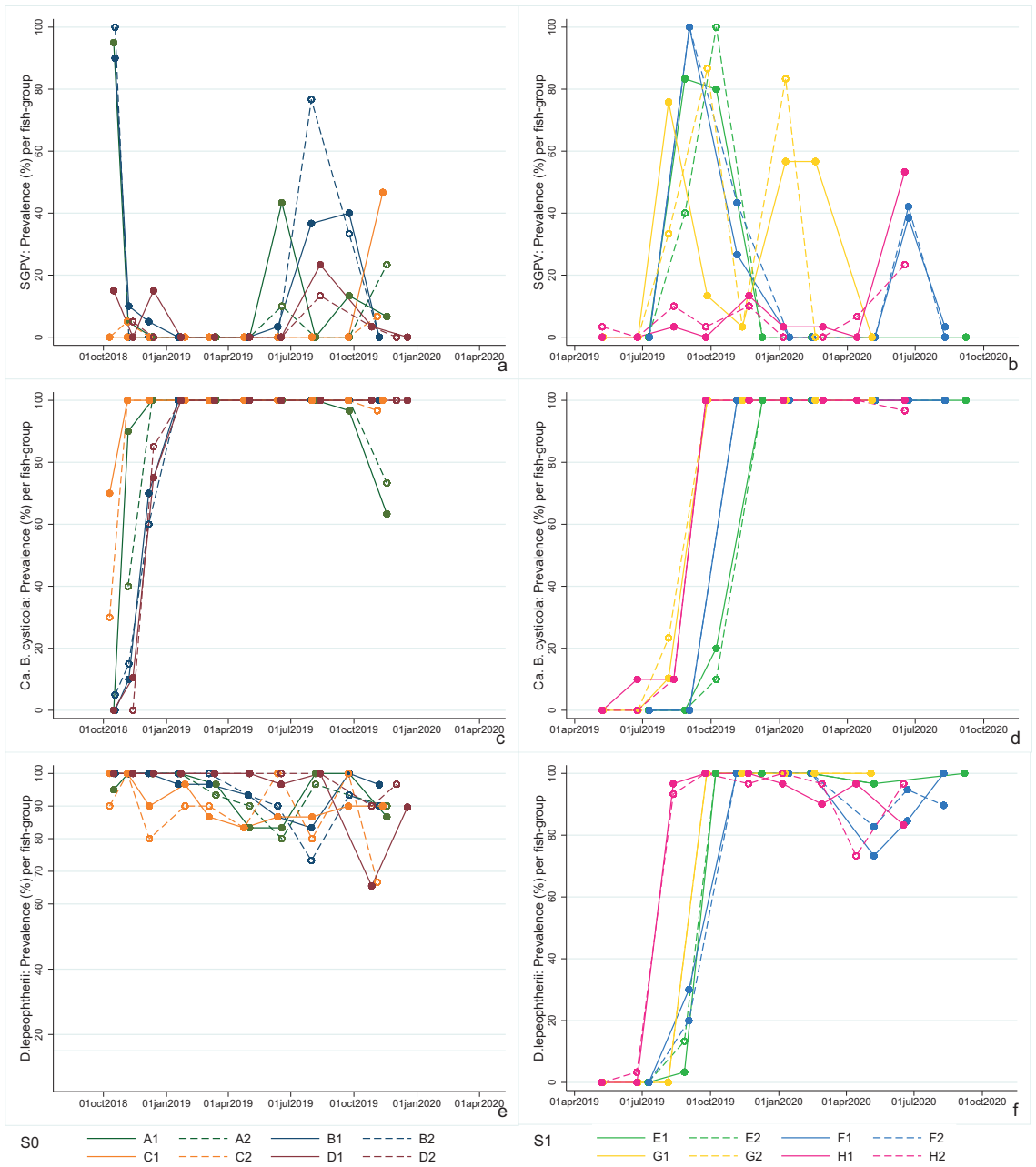
Figure S1 Percentage of RT-qPCR positive fish per fish-group throughout the sea-phase for autumn-transferred (S0) and spring-transferred (S1) fish. a) SGPV (S0), b) SGPV (S1), c) *Ca.* B. cysticola (S0), d) *Ca.* B. cysticola (S1), e) *D. lepeophtherii* (S0), f) *D. lepeophtherii* (S1).

Table S1. Overview of samples collected per sampling point and fish-group. Sampling number 1-3 denote samples collected prior to sea-transfer while sampling number 4-13 occurred after sea transfer. h indicates samples available for histology, p samples available for RT-qPCR-analysis. NS = not sampled.

Group / Sampling no.	A		B		C		D		E		F		G		H	
	A1	A2	B1	B2	C1	C2	D1	D2	E1	E2	F1	F2	G1	G2	H1	H2
1	30 (h) 29 (p)		NS		NS			30	20			30		30		30
2		30	NS		NS			30	30 (h) 59 (p)			30		30		30
3	-		NS	NS	NS	NS				30						
Sum FW	60 (h) 59 (p)		NS	NS	NS	NS		60	50 (h) 79 (p)	30	0	60	0	60	0	60
4	20	NS	20	20	10	10	20 (h) 19 (p)	NS	30	30	30	30	30	30	30	30
5	20	20	20	20	20	20	20	20	30	30	30	30	30	30	30	30
6	20	20	20	20	20	20	20	20	30	30	30	30	30 (h) 29 (p)	30	30	30
7	30	30	30	30	30	30	30	30	30 (h) 27 (p)	30	25	20	30	30	30	30
8	30	30	30	30	30	30	30	30	30	29	29 (h) 28 (p)	26	30	30	30	30
9	30	30	30	30	30	30	30	30	30	30	30	29	30	30	30	30
10	30	30	30	30	30	30	30	30	30	NS	0 (h) 26 (p)	0 (h) 19 (p)	30	30	30	30
11	30	30	30	30	30	30	30	30	NS	NS	30	29	30	30	30	30
12	30	30	30	30	30	30	30 (h) 29 (p)	30	NS	NS	NS	NS	NS	NS	30	30
13	30	30	30 (h) 29 (p)	30	30	30	30 (h) 29 (p)	30	NS	NS	NS	NS	NS	NS	NS	NS
Sum sea	270	250	270 (h) 269 (p)	270	260	250	270 (h) 267 (p)	250	210 (h) 207 (p)	179	204 (h) 229 (p)	194 (h) 213 (p)	240 (h) 239 (p)	240	270	270

Fish-group	A1	A2	E1	E2	B1	B2	F1	F2	C1	C2	G1	G2	D1	D2	H1	H2
#Delousing (all NMT)	1	5	8	7	5	4	3	2	11	10	0	1	7	8	8	8
#Mechancial delousing	0	0	3	3	0	0	1	1	1	0	0	0	1	1	0	0
#Thermal delousing	1	4‡	4	3	1	1	1	0	8	8	0	1	5	6	6	6
#Hydrogen peroxide	0	0	0	0	0	0	0	0	1	1	0	0	1	1	0	0
#Freshwater bath	0	1	1	1	4	3	1	1	1	1	0	0	0	0	2	2
#Azamethiphos bath	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Phytoplankton cells/L(max)	57384.5		18896.9		47094.9		59159.8		732163.7		231016.0		535779.2		311179.7	
Jellyfish #/m^3 (max)	21.7		0.7		19.9		19.3		47.7		15.9		7.2		11.3	
N. perurans	27.0	27.2	24.6	34.6	13.4	26.3	12.7	14.6	8.8	6.0	18.4	32.8	36.8	32.1	4.1	2.2
Ca. B. cysticola	87.4	92.0	59.4	51.4	83.6	83.7	73.8	71.8	98.8	96.8	84.3	90.8	64.0	65.4	68.9	67.4
D. lepeophtherii	94.1	93.2	71.0	68.2	94.8	93.3	72.5	70.4	90.0	87.2	94.8	98.4	62.8	62.5	73.7	73.3
SGPV	14.4	4.0	23.7	23.5	16.7	19.6	21.4	23.9	5.4	1.2	5.2	2.4	25.5	25.8	8.5	6.3
Epitheliocysts	42.2	44.8	32.4	32.4	50.7	55.9	56.9	48.5	33.5	41.2	38.8	33.3	53.7	58.4	24.1	22.6
Trichodina spp.	0.4	0.0	0.0	0.0	0.7	0.7	0.0	0.0	1.2	0.8	2.9	3.8	0.4	1.6	2.2	1.5
Crustaceans	4.4	7.6	1.4	0.6	3.3	3.3	3.4	3.6	4.2	1.6	1.7	1.7	3.0	3.2	5.6	8.9
Filamentous bacteria	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Bacteria, others	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.5	6.2	0.4	0.4	0.0	0.0	0.4	0.0	0.0
Metacercaria	0.4	0.4	1.9	1.7	2.2	3.0	1.0	2.1	0.0	0.4	3.8	0.8	0.0	0.0	0.4	0.0
Ichthyobodo spp.	0.4	0.4	1.0	0.6	0.4	0.7	0.0	0.5	1.2	1.2	3.8	0.8	0.4	0.4	0.0	0.0
Amoeba (AGD) §	9.3	6.8	5.2	12.8	4.4	11.5	11.8	11.3	1.9	0.0	27.5	21.7	6.3	7.2	1.5	1.1
Foreign material †	0.4	0.4	0.0	0.0	0.0	0.4	0.0	1.0	0.8	0.4	0.0	0.0	0.4	0.4	2.2	0.7
Chronic inflammation filament	13.3	12.8	3.3	1.7	8.1	11.9	4.4	5.7	3.8	3.2	0.4	0.4	0.7	3.2	3.3	1.5
Deformity/abnormal filament shape	4.1	5.6	5.7	9.5	6.7	9.6	14.2	10.3	15.4	9.6	6.7	6.7	8.5	8.8	12.2	11.5
Oedema	2.6	4.4	8.6	5.6	1.1	3.0	14.2	10.8	7.3	3.2	5.0	0.8	4.1	2.8	4.1	3.0
Epithelial necrosis/apoptosis	5.2	6.0	4.3	12.3	7.0	11.5	19.1	12.4	5.8	4.4	19.6	10.4	4.3	12.3	5.9	6.3
Thrombi	77.4	80.4	64.3	50.8	58.9	61.9	74.0	69.1	94.2	85.6	87.5	83.8	64.3	50.8	73.7	82.2
Chronic vascular lesions	72.2	72.0	67.1	59.2	53.3	51.9	75.5	74.2	83.5	81.6	85.4	84.6	67.1	59.2	82.2	83.7
Hemorrhage	67.0	66.8	71.9	68.2	73.7	67.0	82.8	80.4	75.8	69.2	82.5	80.0	71.9	68.2	74.8	75.2

Necrosis whole lamella	0.0	0.0	0.0	0.0	0.0	1.1	0.0	1.0	1.5	0.0	0.8	0.0	0.0	0.0	2.2	0.0
Histology lesions ≥5%	3.3	2.8	2.4	0.6	1.9	4.8	2.5	2.1	4.6	1.2	21.7	14.6	0.0	1.6	1.9	0.7
Gross score >1	15.9	13.1	3.8	3.2	16.5	16.2	0.1	0.1	9.0	8.06	4.3	4.6	1.4	1.7	4.7	3.6
Gill-related mortality % accumulated	0.40	0.04	0.10	0.13	0.42	0.41	0.0	0.0	0.0	0.0	1.69	1.17	0.0	0.0	1.49	1.38
Total mortality % accumulated	6.3	7.4	21.6	18.4	16.1	15.6	6.9	13.9	24.4	39.3	11.7	7.4	6.4	16.8	15.0	21.0

Table S2. Overview of results per fish-group. For non-medicinal delousing (NMT) the number of treatment events are listed. The percent fish with the pathogen or lesion is listed for gill pathogens detected or observed, and for gill lesions and gross score. ‡One of these treatment events was a combination of freshwater bath and thermal delousing. §This category includes both the observation of parasites morphologically consistent with amoeba and AGD diagnosed based on presence of these parasites and typical histopathological lesions. Amoeba without histopathological lesions typical of AGD was not observed in any of the fish. [†] The presence of foreign material was only recorded when the material was associated with a tissue reaction as for instance inflammation or hyperplasia.

1   **Supplementary file 2**

2   **Plankton sampling and analysis**

3   Plankton was collected using 250 µm mesh nets, with 25 cm and 50 cm diameter ring for

4   phyto- and zooplankton, respectively. The nets were submerged to 10-meter depth and slowly

5   pulled towards the surface. Once on the surface, water was poured down the outside of the net

6   to further concentrate the sample into the filter. Approximately 300 ml of seawater was used

7   to wash the contents of the filter into the phytoplankton collecting bottle and 5 ml Lugols

8   iodine solution was then added for preservation. The zooplankton samples were immediately

9   fixed in formalin (10%) in 50% solution with seawater. The samples were stored avoiding

10   direct sunlight and at cool temperatures before being shipped to Fish Vet Groups laboratory in

11   Oslo.

12

13   The phytoplankton container was gently rotated to suspend the plankton in solution prior to

14   placing a subsample of 1 ml into a Sedgewick counting chamber slide for assessment under an

15   inverted microscope (Leica DMIL LED). Phytoplankton was identified and grouped as

16   unspined diatoms, *Chaetoceros* spp., *Pseudo-nitzia* spp., *Ceratium* spp., *Rhizosolenia* spp. and

17   others. The total number of cells and the number cells of different species per litre was

18   estimated by counting the number of cells per square. The number of cells per litre was the

19   calculated as follows:

20   $\text{Cells/liter} = \dfrac{\text{average cells per square} \times 1\,000\,000}{\text{sample concentration factor}}$

21   Where:

22   $\text{Sample concentration factor} = (\text{radius of sample net in meters})^2 \times \text{sample depth } \times \pi \times 1000$

23 For zooplankton quantification the container was gently rotated, and subsample of 150 ml was

24 transferred to cell culture bottles for evaluation under a stereomicroscope (Leica S9D). The

25 number of microscopic jellyfish (*Scyphozoa*, *Cubozoa* and *Hydrozoa*) in the subsample that

26 were considered potentially harmful to fish were counted. Then the total number of

27 zooplankton per cubic meter was calculated as follows:

28 $$\text{Organisms/m}^3 = \frac{\text{average number of organisms per subsample} \times (\frac{300}{50})}{(\text{sample concentration factor}) \times 100}$$

29

30

Supplementary file S2: Histopathology assessment

This two-step system was based on first estimating the number of lamella available for evaluation in each sample, and then using this estimate to calculate the estimated percent of gill tissue affected by different lesions.

Step 1. Estimate the number of secondary lamellae in the sample.

In a small subset of fish representative of the samples to be analyzed one filament was measured and the number of lamellae was counted. This filament should have adequate orientation (i.e., lamellae present on at least one side of the filament along the entire length of the filament). Then the number of lamellae per millimeter filament was calculated for each sample (#lamellae/filament length in millimeter) and the mean number lamella per mm filament across all samples for each sample set was used in further calculations to estimate the number of lamella available for examination in each gill sample.

For each sample to be scored the number of filaments was counted and a filament deemed representative of the mean filament length in the sample was measured. All filaments were then assessed for quality of plane of section and the number of filaments with good, average, and poor plane of section was counted. For a filament with good plane of section lamellae were present on both sides along the majority (> 80%) of the filament. In filaments with average plane of section lamellae were present on both sides along 50 to 80% of the filaments, while lamellae were present on both sides on less than 50% of the filament as poor plane of section. Based on the above information, the estimated number of lamellae available for assessment in each sample was calculated as follows:

1. #Lamella per filament $=$ length of average filament (mm) $\times$

   $mean\ \#lamella\ per\ mm\ filament$

2. $\#Lamella\ in\ sample = (\#filament\ in\ good\ plane\ of\ section\ \times$

   $\#lamella\ per\ filament) + (\#\text{filaments in average plane of section}\ *$

   $\#\text{lamellae per filament} * 0.8) + (\#\text{filaments in poor plane of section}\ *$

   $\#\text{lamellae per filament} * 0.5)$

Step 2. Estimating the % gill tissue affected by a given gill lesion.

For each of the categories of gill lesions that were recorded quantitatively and semi-quantitatively the number of lamellae affected by each lesion were counted. The percent of affected lamellae was subsequently calculated as:

$$Percent\ affected\ lamellae\ = \frac{number\ of\ lamellae\ with\ a\ lesion}{estimated\ number\ of\ lamellae\ in\ the\ sample} \times 100$$

Counts and percent of lamellar epithelial hyperplasia and/or hyperplasia and inflammation, acute vascular lesions, non-acute vascular lesions (thrombi, reactive and reparative vascular lesions), lesions with overlap of hyperplasia and vascular lesions, and necrosis was recorded. These lesions were defined as follows:

Acute vascular lesions

  o Aneurysms, i.e., dilation of lamellar vascular sinusoids with rupture and loss of pilar cells, but without proliferation of lamellar epithelium, fibrin deposition or thrombosis of the affected vessel.

Non-acute vascular lesions

- o Aneurysms, with fibrin deposition, and with and without proliferation of lamellar epithelium or infiltration of intact cells into the thrombosed vessel.

- o Thrombi in lamellar sinusoids with normal diameter, with or without proliferation of lamellar epithelium or infiltration of intact cells into the thrombosed vessel.

- o Recanalization of thrombus or infiltration of fibrovascular tissue with or without obliteration of the vessel lumen.

Hyperplasia

- o Proliferation of lamellar epithelium cells to the extent that at least 80% of the interlamellar space was filled with hyperplastic cells. Lamella on each side of the affected interlamellar spaces were counted.

- o Hyperplastic tissue infiltrated with leukocytes or subepithelial inflammation and hyperplasia, e.g., a combination of inflammation and hyperplasia, was also recorded as hyperplasia.

Necrosis

- o Cell death of a least one secondary lamella, including epithelium and pilar cells.

In addition, presence or absence of the following lesions and presence of any pathogens were recorded as 0 or 1:

Haemorrhage

- o Extravascular erythrocytes within the gill tissue. Red blood cells free between lamella or filaments were not counted as hemorrhage.

Lamellar oedema/"lifting"

- Accumulation of eosinophilic homogenous or granular material (presumed fluid) between the lamellar basal lamina and lamellar epithelial cells.

Epithelial cell necrosis or apoptosis

- Lamellar epithelial cells with hypereosinophilic cytoplasm and/or nuclear pyknosis, karyorrhexis or karyolysis
- Lamellar epithelial cells with margination or clumping of nuclear chromatin

Chronic inflammation of the filaments

- Severe inflammation expanding the filament with infiltration of inflammatory cells and/or fibrosis of filament supportive tissue clearly visible on low magnification.
- May be associated with hyperplasia, vascular lesions, or loss of overlying lamella.

Deformed filaments

- Filaments with abnormal shape irrespective of presumed cause.
  - Thickened and proliferative filament cartilage and/or bone
  - Branching filaments
  - Shortened filaments
  - Deviation of filament direction

Amoebic gill disease (AGD)

- Segmental lamellar epithelial hyperplasia and presences of parasites morphologically consistent with amoeba.

Foreign material

- o Foreign material present between lamella associated with tissue reaction (inflammation, necrosis, hyperplasia, and/or vascular lesions)

II **Assessment of acute effects of in situ net cleaning on gill health of farmed Atlantic salmon (*Salmo salar* L)**

Assessment of acute effects of *in situ* net cleaning on gill health of farmed Atlantic salmon (*Salmo salar L*)

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ABSTRACT

Regular *in situ* cleaning of net pens is performed to prevent overgrowth and negative effects of biofouling during the sea phase of salmon production in Norway. Possible negative health effects of *in situ* net cleaning include a reduction in appetite, increased stress, and gill damage. Gill lesions have been reported after exposure to *Ectopleura larynx* in laboratory trials, but there is currently little information available on whether exposure to biofouling debris from *in situ* net cleaning significantly contributes to development of gill disease and reduced gill health of farmed Atlantic salmon (*Salmo salar*) in sea cages. To assess this, gill tissue from healthy fish ($n = 30$ per pen/time point) from 3 net pens were collected before, as well as one and eight days after the first net cleaning event after sea transfer. The tissue was examined using a standardized histology scoring system. All fish originated from the same commercial sea site located in North-western Norway and net cleaning was performed as per routine for this site. The level of net fouling was scored using a semiquantitative scoring system ranging from 0 to 6, *i.e.*, from totally clean to heavy fouling. Two pens were moderately fouled, and one had a low degree of fouling. The probability (odds) of fish from moderately fouled net pens having subacute vascular damage (thrombi) in the gills at one day after net cleaning was 2.36 (95% CI 1.21–5.71) times higher compared to fish sampled before net cleaning. When all fish were included in the statistical analysis no difference in the number of fish with the recorded lesions were found across the different sampling points and there was even a small decrease in the probability (odds) of fish having acute vascular lesions at eight days after net cleaning. Our results suggest that exposure to biofouling debris during net cleaning might contribute to development of thrombi/subacute vascular lesions in the gills. However, the proportion of gill tissue affected was low, generally estimated to be less than 1%, and no significant difference in the number of fish with these lesions was found at eight days after net cleaning. Thus, the negative impact of *in situ* net cleaning on gill health in this study is small and short lived, and the clinical implication of these results remains to be established.

1. Introduction

In finfish aquaculture, biofouling is defined as unwanted accumulation of living organisms on submerged artificial surfaces (Bloecher, 2013; Wahl, 1989). Biofouling has several negative effects on salmonid aquaculture (Bannister et al., 2019; Bloecher and Floerl, 2020a; De Nys and Guenther, 2009; Fitridge et al., 2012). High levels of biofouling can lead to deformation and damage to the nets due to the increased weight and drag caused by the fouling organisms (Gansel et al., 2015; Lader et al., 2015; Swift et al., 2006). Biofouling occludes net apertures and can limit the water exchange and reduce oxygen levels within a pen (Braithwaite et al., 2007; Cronin et al., 1999; Madin et al., 2010). It has been suggested that performance of cleaner fish can be reduced as biofouling can serve as an alternative food source replacing salmon lice (Imsland et al., 2015; Kvenseth, 1996). However, a later study found a positive effect of the presence of biofouling on cleaner fish efficacy (Eliasen et al., 2018). Multiple fish pathogens have been identified in biofouling organisms and within biofouling communities (Albert and

Ransangan, 2013; Douglas-Helders et al., 2003; Hellebo et al., 2017; Napsøy, 2020; Pietrak et al., 2012), but if biofouling communities are significant reservoirs for pathogens or if biofouling debris can serve as vectors for pathogen transmission in salmonid aquaculture remains to be established.

Fish-farming companies most often employ a combination of prevention methods to mitigate the negative effect of biofouling, using biocidal net coatings and removal of biofouling by *in situ* cleaning of nets (Bloecher and Floerl, 2020a). Physically changing fouled nets and cleaning them on land after removal is less common in Norwegian aquaculture but is practiced by some companies and in other regions (Bloecher and Floerl, 2020a). Copper oxide, either alone or in combination with other compounds is the main biocide used, although more environmentally friendly alternatives exist (Bloecher and Floerl, 2020b; Edwards et al., 2015). Remotely operated cleaning rigs or remotely operated vessels fitted with rotating discs generating water jets flushing the biofouling off the nets are used for *in situ* net cleaning. The cleaning pressure used ranges from 50 to 350 bar (Bloecher et al., 2019). In regions of Norway with high levels of biofouling an average of 15 cleaning events are required per production cycle (Bloecher and Floerl, 2020a). Particles generated during cleaning are released to the water column and fish in the pen being cleaned as well as fish in pens downstream can be exposed to the debris (Carl et al., 2011; Floerl et al., 2016). This debris mostly consists of biofouling organisms (Bloecher et al., 2019; Carl et al., 2011), but could also contain particles of antifouling coating (Bloecher et al., 2019; Floerl et al., 2016; Napsøy, 2020).

Net cleaning can lead to a temporary drop in appetite in farmed salmon, which could impact growth rate, particularly if frequent *in situ* net cleaning is performed (Bloecher and Floerl, 2020a). It has been suggested that stress induced by net cleaning (Stene et al., 2018) could trigger disease outbreaks (Bloecher and Floerl, 2020a) and injuries to cleaner fish after net cleaning have been reported (Imsland and Nytrø, 2017). Lastly, a direct negative impact of exposure to biofouling debris, mainly from the hydroid *Ectopleura larynx* (syn. *Tubularia larynx)*, has been reported (Baxter et al., 2012; Bloecher et al., 2018; Comas et al., 2021; Fisher and Appleby, 2017). The hydroid *E. larynx* and other cnidarians may cause damage due to nematocysts on hydroid fragments firing and releasing toxins when they come into contact with fish surfaces such as skin and gill (Baxter et al., 2012; Bloecher et al., 2018; Helmholz et al., 2010). Histologic lesions observed after exposure to cnidarians include gill thrombi, haemorrhage and necrosis, lamellar epithelial hyperplasia and inflammation, and lamellar oedema/lamellar epithelial lifting (Baxter et al., 2011a; Baxter et al., 2012; Baxter et al., 2011b; Bloecher et al., 2018; Marcos-Lopez et al., 2016; Mitchell et al., 2011; Powell et al., 2018).

There is currently little information available on whether exposure to biofouling debris from *in situ* net cleaning significantly contributes to development of gill disease and reduced gill health of Atlantic salmon farmed in sea cages in a commercial setting. Similarly, information on which biofouling strategies are optimal for gill health are lacking. Napsøy (2020) examined gills of Atlantic salmon and rainbow trout (*Oncorhynchus mykiss*) from one pen at three different farms before and after net cleaning but found no clear difference in gill lesions between different sampling points. To the best of our knowledge there have not been any other studies published examining the effect of *in situ* net cleaning on gill health in commercial sea farms.

The aim of this study was to examine if *in situ* net cleaning can have an acute, direct impact on the gill health of farmed Atlantic salmon. To assess this, gill tissue from presumed healthy fish was collected before and after the first net cleaning event after sea transfer and examined using a standardized histology scoring system. More knowledge about potential effects of net cleaning on fish health will be helpful to make informed decisions about biofouling management.

2. Material and methods

2.1. Study population

The study site was a commercial sea site in Northwest Norway in Møre og Romsdal county and consisted of six pens with Atlantic salmon. Of these, three pens were included in the study. The pens and site were selected based on the following criteria: a) The fish in the study pens had not been deloused and the nets had not been washed since the fish arrived at the site. b) There were no known gill health issues, issues related to plankton or jellyfish or other diseases at the site. c) Three pens with fish matching the previous criteria could be cleaned on the same day. d) No other pens at the site would be cleaned during the study period. e) Fish in the study pens were as similar as possible (timing of sea transfer, genetics *etc.*). The site is shielded from the open sea by an island and has intermediate exposure to currents and waves when compared to sites within a fjord (low exposure) or completely exposed to the open ocean (high exposure). The main current at the site moves in a northeast direction. Fish originated from Aquagen roe and were all from the same hatchery and were sea-transferred within five days in September 2020. The month before net washing sea temperature ranged from 9.5 to 12.1 °C and oxygen saturation ranged from 80.3 to 96.3%. Salinity >30‰ and there were no significant fluctuations in salinity level at the site. The daily mortality at the site was low, never exceeding 0.01% for the study pens throughout November and December 2020. No clear difference in daily mortalities were observed at the time of net cleaning when compared to the month before. Similarly, no clear reduction in specific feed rate (SFR) or specific daily growth rate (SGR) was observed at the time of net cleaning (data not shown). Mean fish weight, fish number and biomass per pen on the treatment day is summarized in Table 1. Lumpfish (*Cyclopterus lumpus)*, ballan wrasse (*Labrus bergylta*) and goldsinny wrasse (*Ctenolabrus rupestris*) had been added to all pens for biological lice control.

Fish were kept in multifilament nylon nets with small to medium mesh-size of 15.5-mm half-mesh and aperture of 27.4 mm. The nets were coated with Netwax GreenlineE5 (Netkem) which contains 25–30% copper oxide (Cu$_2$O and CuO). The distance between pens were a minimum of 140 m and pens were circular with a circumference of 135 m and total volume of 27,699 m^3. Net washing and sampling were performed in late November 2020. All three project pens were washed on the same day using a Manta net cleaner (Stranda Prolog). The Manta net cleaner is remotely operated and works inside the pen by flushing the biofouling out of the net with waterjets generated from five rotating nozzles. The flushing pressure used during washing ranged from 80 to 90 bar for the bottom of the nets and 120 bar for the walls. Cleaning time ranged from approximately 80 min (pen C) to 120 min (Pen A and B). Pen B was cleaned first, followed by pen C and pen A, (see Fig. 1. for outline of the farm). Pen B is furthest to the southwest with the site, followed by pen A, while pen C is at the northwest aspect of the farm. Debris from cage B could be brought by the current to pen A and C, while debris from pen A could flow through pen C. The sea current during washing was considered moderate to marked, but exact current velocity was not measured or recorded.

2.2. Scoring of net biofouling

Photos of each pen were captured prior to net washing using a

Table 1
Mean fish weight, fish count and biomass for each pen at the day of net cleaning.

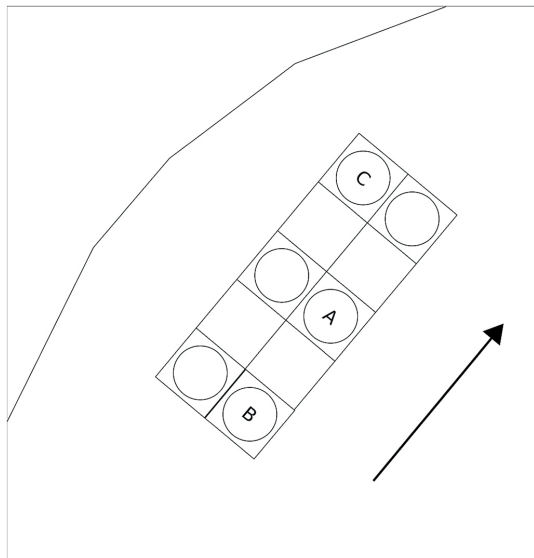| Pen	Mean fish weight (gram)	Fish count	Biomass (kg)
A	731.2	158,810	116,127
B	704.4	161,189	113,-536
C	546.6	184,355	99,128

Fig. 1. Outline of the sea farm and study cages A-C. The arrow represents the direction of the main current at the site.

remotely operated vehicle (ROV). Three photos were taken of different parts of the nets at four depths, 1, 5, 10 and 29 m below the surface, but photos at 1- and 5-m depth were not available from Pen A and C, respectively. For each depth, a mean score was calculated for three images as well as a mean score for all available images per pen (Table 2). The level of net fouling was scored using a semiquantitative scoring system developed by Mowi Canada West (personal communication Rodrigo Cristi, Sandra Huynh, and Bogdan Vornicu) ranging from 0 to 6, *i.e.*, from totally clean to heavy fouling. The scoring was based on the proportion of the net covered with biofouling. In addition, the fouling was assessed by the operators during net washing using a semi-quantitative scale ranging from none, sparse, moderate to marked. A score was assigned to the walls and the bottom for each net pen. It was not possible to identify the species of fouling organisms present due to the quality of the photos and the distance from the ROV to the net when the image was captured. However, farm staff had observed *E. larynx* growing on the nets. In January 2021, a sample of net fouling was collected by scraping the net, placed in formalin, and sent to Fish Vet Group Norge. The fouling material was examined using a stereomicro-scope and *E. larynx* was identified, confirming the presence of this or-ganism at the farm (Fig. 2.) (Hayward and Ryland, 1990).

Table 2
Results of net scoring of net photos and scores from net cleaning operator. Im-ages of the net was scored from 0 to 6, while the person operating the net cleaner scored from 0 to 3. ND – not done, images not available.

Pen	Score 1 m Mean Min - Max	Score 5 m Mean Min - Max	Score 10 m Mean Min - Max	Score 29 m Mean Min - Max	Score Overall Mean Min - Max	Score operator Net walls	Score operator Net base
A	ND	4 1–6	2.67 1–4	2.67 1–4	3.11 1–6	2	1
B	4 2–6	1.5* 0–3	3.33 1–6	1.33 1–2	2.54 0–6	2	1
C	1.33 1–2	ND	0.67 0–1	0.67 0–1	0.89 0–2	1	0

* Only two images available for scoring at this depth.

Fig. 2. Stereomicrograph of a hydroid polyp in a formalin fixed sample of fouling material. Bar 1 mm.

2.3. Sampling

Thirty fish per pen were sampled one day prior to net washing, as well as one day and eight days after net cleaning, respectively. The aim was to sample up to 15 fish with clinical signs of gill disease and 15 healthy fish per pen after net washing; however, no moribund fish or fish with overt signs of gill disease were observed when sampling after net washing so all sampled fish were apparently clinically healthy. One moribund fish was included in the sampling prior to net washing. The cause the clinical signs observed in this fish was not determined, and minimal lesion was observed in the gill tissue. No gross gill lesions were observed prior to net washing and minimal to mild gill lesions (white areas) were observed after net washing. Systematic gross examination of gills and scoring of gross lesions was not performed. Euthanasia was achieved with an overdose of benzocaine (Benzoak vet, ACD Pharma-ceuticals AS, Leknes, Norway). Within five minutes after euthanasia the second left gill arch was sampled for histology.

2.4. Histology

Gills were fixed in buffered formalin (4% formalin, 0.08 M sodium phosphate, pH 7.0), processed in a Thermo Scientific Excelsior tissue processor (Thermo Fisher Scientific, Runcorn, UK) and embedded in paraffin using a Tissue – Tek, TEC 5 (Sakura Finetek Europe B.V., Alphen aan den Rijn, The Netherlands) embedding centre. Embedded tissue was sectioned at 1.5–2 μm using a Leica RM 2255 Microtome, sections were mounted on glass slides and stained with haematoxylin-eosin (HE). Stained slides were scanned in an Aperio ScanScope AT Turbo slide scanner and read using Aperio ImageScope (Leica Biosystems, Buffalo Grove, IL, United States). All counting and measurements were done using the annotation tools in the ImageScope software. Slides were randomized using computer generated random numbers, and the pathologist was "blinded" regarding pen and time point of sampling relative to net cleaning.

A two-step scoring system was used. First the number of lamella available for evaluation in each sample was estimated, and then all affected lamella with a given lesion were counted. These counts were used to calculate the estimated percent of gill tissue affected for each type of lesion (Appendix A: Histology score system). Counts and percent affected gill tissue were recorded for the following lesions (Fig. 3.):

- Acute vascular lesions (comprising of haemorrhage and acute telangiectasis/aneurysms)
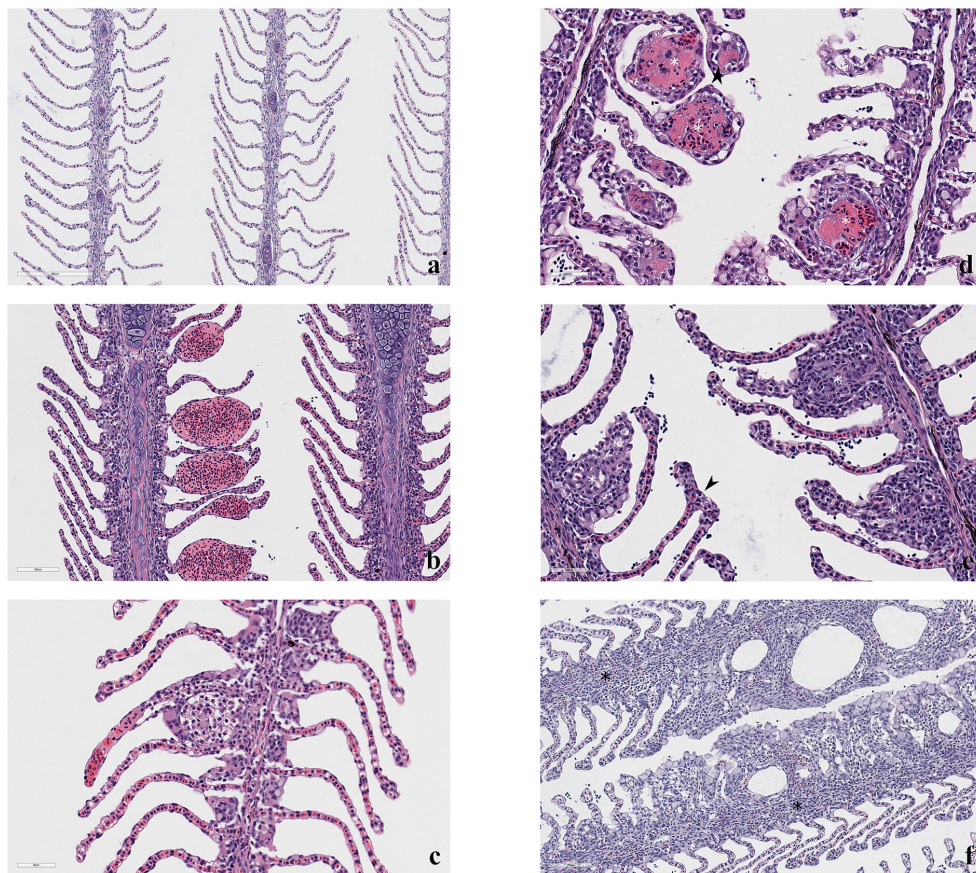
Fig. 3. Normal gill tissue and histology lesions. All tissues stained with haematoxylin and eosin.
a) Normal gill tissue. b) Acute aneurysms. c) Subacute vascular lesion – a thrombus, and focal inflammation. d) Early chronic vascular lesions - thrombosed aneurysms with lamellar epithelial hyperplasia (*) and presumed recanalization (arrowhead). e) Late chronic vascular lesions – orderly lamellar vascular sinusoids replaced by fibrovascular tissue (*). The surface of the lesion shows epithelial hyperplasia and goblet cell hyperplasia. Also note lamellar adhesion (arrowhead). f) Lamellar epithelial hyperplasia. Also present are lamellar fusion and vesicles, goblet cell hyperplasia, haemorrhage, and inflammation of the filaments (*) and inflammatory infiltrates beneath the lamellar and interlamellar epithelium.

- Subacute vascular lesions (comprising of thrombi and thrombosing aneurysms/telangiectasia)
- Chronic vascular lesions (organizing thrombi and aneurysms)
- Hyperplasia
- Necrosis (of at least one lamella)

In addition, presence or absence of the following lesion were recorded as 0 or 1:

- Epithelial cell necrosis
- Adhesion of lamella
- Hypertrophy/swelling of epithelial cells
- Lamellar oedema/"lifting"
- Inflammation in the filaments
- Subepithelial inflammation

Any pathogens observed in the tissues were also recorded as present or absent. In addition, inflammation was also assessed by examination of 3 filaments at high magnification, counting 10 lamella per filament and recording how many of these were infiltrated with presumed inflammatory cells.

2.5. Statistical analysis

Statistical analysis of results was undertaken in STATA (StataCorp. 2015. Stata Statistical Software: Release 14. College Station, TX, United States). Box and whisker plots were produced to provide a visual presentation of the percent gill tissue affected by parameters hyperplasia, acute, subacute, and chronic vascular lesions per pen and time point (Fig. 4.). Percent affected tissue was used for the visual presentation as the calculated percentage considers the amount of tissue available for examination in each sample, while the recorded counts per sample do not. The variance of the lesion counts was considerably greater than the mean and there was an excessive number of samples with zero counts. Counts were therefore transformed into dichotomous variables. To determine if there was as significant change in the number of fish with different gill lesions before and after net washing, we used a series of logistic regression models with each category of lesion as the response
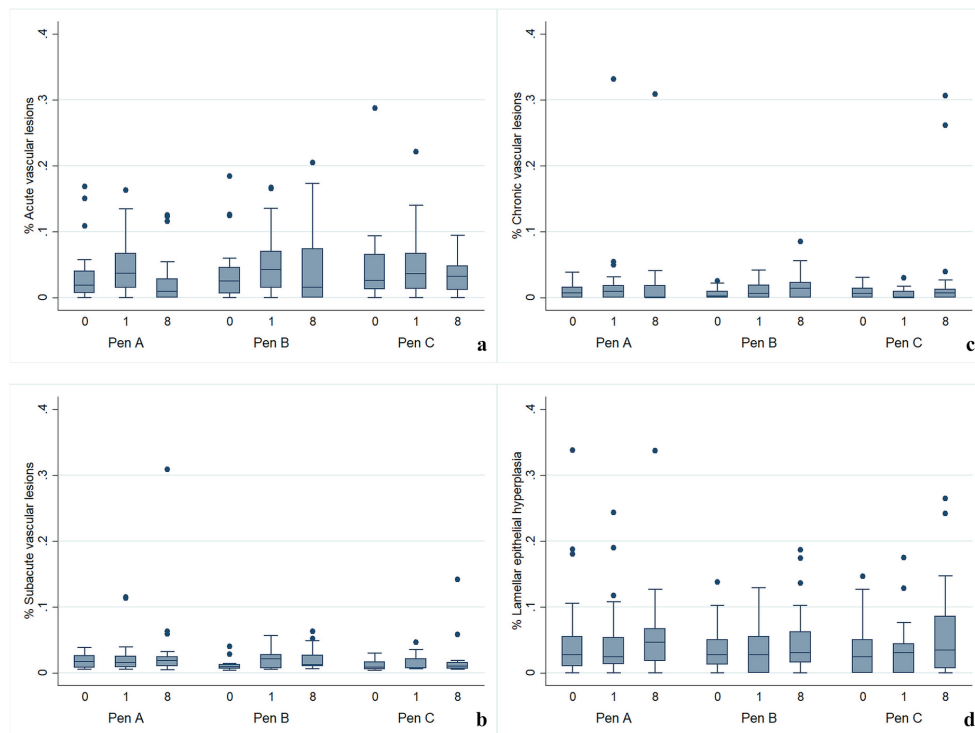
Fig. 4. Box and whisker plots show percent affected gill tissue per pen and timepoint (day) ($n = 30$) for: a) acute vascular lesion (haemorrhage and acute aneurysms), b) subacute vascular lesions (thrombi and thrombosed aneurysms), c) chronic vascular lesions (repairing and reorganizing thrombi and thrombosed aneurysms), d) lamellar epithelial hyperplasia. For clarity one outlier were excluded from plots of acute and chronic vascular lesions and three outliers were excluded from the plot of percent hyperplasia.

variable and sampling point and cage as predictor variables. As the level of biofouling was mild for cage C and as we might not expect an effect of net cleaning for fish in this cage, separate statistical analysis was also performed for cage A and B only. The odds ratio (OR) was calculated for the two time points after net cleaning. Odds ratio represents the odds that the fish will have a given lesion after net washing compared to the odds of having this lesion before net washing. An OR of 1 means there is no difference between the fish sampled before and after. An OR higher than one means an increase in the number of fish with a given lesion after treatment and an OR lower than 1 means a decrease in the number of fish with a given lesion after treatment. For all analyses differences were considered significant at a probability level of $p < 5\%$.

3. Results

3.1. Net scoring

Results of net scoring are summarized in Table 2. The fouling level of pen C was low, with an overall mean score of 0,89 and a maximum score of 2, while the fouling levels of pen A and B were moderate. Mean overall score for pen A and B was 3.11 and 2.54 with scores ranging from 1 to 6 for pen A and 0–6 for pen B. This was in agreement with the assessment of the net washing operator, who also found mild (pen C) to moderate fouling (pen A and B) on net walls and none (pen C) to mild fouling (pen A and B) on the net bases.

3.2. Histology

In general, very few lesions were observed in the gills both before and after net washing, consistent with overall good gill health. The number and percent of fish with different lesions, median, minimum, and maximum lesion counts per sampling point are summarized in Table 3. The number of lamellae per sample across all pens affected by any vascular lesion before net washing ranged from 0 to 58, with a median of 6.5 before net washing. At one day after net cleaning the number of affected lamellae ranged from 0 to 216, with a median of 9, while at eight days after the range was 0 to 67 with a median of 6. The number of lamellae with hyperplasia ranged from 0 to 126 before, 0 to 39 one day after and 0 to 347 eight days after net cleaning, with a median of 4 for all time points. Amoebae and segmental hyperplasia with formation of so-called vesicles consistent with amoebic gill disease (AGD)-type histopathology were found in four fish. Amoeba were observed in one gill from pen A and two gills from pen C prior to net cleaning, and in one fish from pen A eight days after net cleaning. The gills with higher counts and percent of hyperplasia, were generally the gills with AGD-lesions. Intracellular bacteria (epitheliocysts), parasites consistent with trematode metacercaria and crustaceans were found in three, one and two gills, respectively.

3.3. Statistical analysis

When including all pens in the statistical model there was no statistically significant difference in the number of fish with subacute and

Table 3

Summary of histology lesions for all pens across the three sampling points. The first column per time point contains the number and percent of fish with a given lesion. The second column contains the median, minimum and maximum number (counts) of lesions observed in the gill tissues of the examined fish per sampling point. ND = not done.

Gill lesions	Before		1 day after		8 days after	
	# fish % fish	# of lesions median min-max	# fish % fish	# of lesions median min-max	# fish % fish	# of lesions median min-max
Acute vascular lesion	79 88%	4 0–53	81 90%	6 0–59	63 70%	2 0–26
Subacute vascular lesion	46 51%	1 0–9	55 61%	1 0–18	56 62%	1 0–33
Chronic vascular lesion	53 59%	1 0–6	48 53%	1 0–203	53 59%	1 0–39
Sum vascular lesions	88 98%	6.5 0–58	86 96%	9 0–216	81 90%	6 0–67
Hyperplasia	69 80%	4 0–126	69 77%	4 0–39	73 81%	4 0–347
Diffuse inflammation	64 71%	2 0–10	67 74%	2 0–16	65 72%	2 0–17
Epithelial cell necrosis	32 36%	ND	30 33%	ND	23 26%	ND
Epithelial hypertrophy/ swelling	6 7%	ND	2 2%	ND	1 1%	ND
Lamellar adhesion	30 33%	ND	29 32%	ND	38 42%	ND
Lamellar oedema/ "lifting"	2 2%	ND	0 0%	ND	1 1%	ND
Inflammation filament	2 2%	ND	3 3%	ND	2 2%	ND
Subepithelial inflammation	9 10%	ND	11 12%	ND	4 4%	ND

chronic vascular lesions, diffuse inflammation, or hyperplasia across the different sampling points. In addition, there were no difference in the number of fish with epithelial cell necrosis, epithelial hypertrophy, lamellar adhesions, lamellar oedema, or inflammation of the filaments or lamella. Acute and subacute vascular lesions showed a significant difference between timepoints when statistical analysis was repeated for pen A and B only. There was an increase in the number of fish with subacute vascular lesions, primarily lamellar thrombi, at one day after net washing (odds ratio = 2.36, se = 1.04, P > |z| = 0.014) in these two pens. Across all pens there were significantly less fish with acute vascular lesions at eight days after net washing compared to before net washing (OR = 0.32, se = 0.13, P > |z| = 0.004).

4. Discussion

The probability (odds) of fish having subacute vascular damage (thrombi) in the gills at one day after net cleaning was 2.36 times higher compared to fish sampled before net cleaning. However, this difference was only found for fish in the moderately fouled pens. When all fish were included in the statistical analysis no difference in the number of fish with the recorded lesions were found across the different sampling points and there was even a small decreased probability of fish having acute vascular lesions at eight days after net cleaning. Overall, our results suggest that exposure to biofouling debris during net cleaning of moderately fouled net pens may contribute to development of thrombi/ subacute vascular lesions in the gills. However, the percent of gill tissue affected was very low, generally estimated to be less than 1% (Fig. 4.), and no significant difference in the number of fish with these lesions was found at eight days after net cleaning. Thus, the negative impact on gill health in this study is small and short lived, and the clinical implication of these results remain to be established.

There is a paucity of field studies examining the effect of *in situ* net

cleaning on gill health, as such there are few other studies to compare our results with. In a recent field study gills of Atlantic salmon and rainbow trout from three farms were examined before and after net cleaning and no clear differences in gill lesions between different sampling points were found (Napsøy, 2020). In this study 2 different net cleaning devices and low to moderate cleaning pressure (60–100 bar) were used. The biofouling communities were dominated by *E. larynx* in two of the three sites. The lack of systematic registration of histologic lesions and the presence of amoebic gill disease in two of the three sites sampled may have limited the ability to detect differences between fish sampled before and after cleaning (Napsøy, 2020).

The finding of a higher prevalence of subacute vascular lesions (thrombi) in gills after net cleaning is supported by one of two laboratory trials where gill lesions were described after exposure to hydroids. Bloecher et al. (2018) found higher non-specific gross gill scores and higher prevalence of thrombi (scores >0) up to one day after exposure, and a higher average number of gill lamellar thrombi from one to seven days after exposing Atlantic salmon to hydroid cleaning waste. Baxter et al. (2012) exposed Atlantic salmon to hydroid colonized net baskets or loose hydroid fragments and scrubbed net baskets and reported focal areas of epithelial sloughing, necrosis, and haemorrhage, but not lamellar thrombi, in both exposed and control groups. The finding of lesions in the control group was attributed to fragments of *E. larynx* entering the water circulation and subsequently control tanks. Both the current study and Bloecher et al. (2018) found an increased prevalence of thrombi at one day after exposure to biofouling debris, but no difference in the number of fish with epithelial necrosis or sloughing across the time points was found in the current study, even when the statistical analysis of only moderately fouled pens was performed. A possible explanation for this discrepancy could be that the lesions reported by Baxter et al. (2012) were unrelated to hydroid exposure as they were found in both exposed and control groups.

In a recent experimental study, Comas et al. (2021) placed gilthead seabream (*Sparus aurata*) juveniles in small net cages in a sea pond and performed regular *in-situ* net cleaning. Fish were followed for a 7-month period, and at the end of the period and prior to the last net cleaning event mean sum histology gill scores (Mitchell et al., 2012) for the cages that had been washed were higher than the mean sum gill score of fish sampled at the start of the experiment. In addition, the authors compared mean sum histology gill scores in fish sampled just before, just after and 24 h after the last net cleaning event and found an increase in mean sum gill scores after cleaning. Development of lamellar thrombi or aneurysms during the study were not mentioned. However, this experimental setup, the environmental conditions and the fish groups used differ significantly from the conditions at commercial salmon sea farms in North-western Europe. In addition, some of the histopathological lesions reported in the study can be related to water quality, sampling, and sample preparation (Speare and Ferguson, 1989; Wolf et al., 2015). Because of this it is uncertain whether these results are applicable for net cleaning in commercial salmon sea farms.

The studies examining potential impact of net cleaning on gill health have primarily focused on effects of exposure to hydroids (Baxter et al., 2012; Bloecher et al., 2018). However, it is possible that particles from other fouling organisms and parts of antifouling coating released during cleaning could have a negative effect on gill health and could contribute to the development of thrombi in the current study and the increased gill score reported by Comas et al. (2021). But the concentration and type of particles generated during net washing were not examined in either study and it is unclear if the concentration, duration, frequency, and characteristics of suspended solids generated by *in situ* net cleaning could impact gill and overall fish health. An increased concentration of suspended solids in the water column has been shown to have negative effects on health and behaviour of salmonids and other fish species (Bilotta and Brazier, 2008; Kemp et al., 2011; Kjelland et al., 2015). High concentrations of particles have been reported to "clog" gills and can lead to mortalities of the affected fish (Cordone and Kelley, 1961). Gill

lesions have been reported in reef fish larva and juveniles exposed to sediments (Australian bentonite and others), including mucous cell and lamellar epithelial hyperplasia, lamellar epithelial lifting, and lamellar shortening (Cumming and Herbert, 2016; Hess et al., 2015; Hess et al., 2017). Other studies have shown that fish exposed to certain mineral particles (kaolin clay, topsoil, volcanic ash, and mica) over time did not develop gill lesions, even if other negative effects on fish health were evident (Michel et al., 2013; Redding et al., 1987). Napsøy (2020) reported an increase in turbidity measurements and sediments to a maximum of 4.5 FTU (formazin turbidity units) and 22% sediment in water samples collected during and immediately after *in situ* net cleaning, but turbidity and sediment levels dropped rapidly when cleaning was complete, and as mentioned above no impact on gill health was evident in that study.

It has been suggested that copper released from antifouling paint by leaching or during *in situ* net cleaning can have negative effects on farmed fish and gill health (Bloecher and Floerl, 2020b; Burridge and Zitko, 2002; Kalantzi et al., 2016). Increased concentrations of total dissolved and labile copper in seawater have been related to handling of nets treated with copper-based antifouling paint (Kalantzi et al., 2016) and copper fragments and antifouling coating particles have been found among water sample sediments after *in situ* net cleaning (Bloecher et al., 2019; Napsøy, 2020). Studies on potential bioaccumulation of copper released from antifouling paint are conflicting (Comas et al., 2021; Nikolaou et al., 2014; Solberg et al., 2002) and histologic gill lesions ascribed to copper toxicity are not specific for this insult (Al-Bairuty et al., 2013; Malhotra et al., 2020). In the current study no measurement of copper concentration in water or tissue samples were performed and any potential effects of copper exposure during net cleaning is unknown. However, increasing water salinity protects fish against toxic effects of copper, rendering copper significantly less toxic in seawater compared to freshwater (Linbo et al., 2009; Sommers et al., 2016). In addition, the form of copper used in antifouling paint has poor solubility in water (Budavari, 1996), indicating that the likelihood direct toxic effects of copper and copper particles released during *in situ* net cleaning on gill health is low.

Fish with no apparent gill disease and no previous exposure to net cleaning and mechanical or thermal delousing were included in the study. Sampling was performed over a limited time span and all fish groups enrolled in the study were from one site. This was undertaken to have the best chance of detecting lesions related to biofouling exposure and reduce the likelihood of other insults that could lead to the development of gill lesions in the study period. However, as this is a field trial environmental factors and pathogens present in the population might affect the outcome of this study. Mild lesions consistent with amoebic gill disease (AGD) were found in a small number of fish, and the presence of amoebae may have affected our ability to detect differences in the number of fish with hyperplasia between timepoints. In contrast, vascular lesions are not typically associated with AGD (Adams and Nowak, 2001) and presence of amoebae in the fish population is not likely to affect the severity of vascular lesions or the number of fish with these lesions. But it is important to note that none of the recorded gill lesions are specific for exposure to hydroids or net cleaning debris, as different insults can lead to similar tissue damage and reactive and reparative responses in the gill (Gjessing et al., 2019; Powell et al., 2018; Wolf et al., 2015).

The splitting of vascular lesions based on perceived chronicity in this study was done to be able to exclude lesions that might develop during capture and handling of fish for sampling and euthanasia (acute aneurysms) (Wolf et al., 2015) from vascular lesions (thrombi, thrombosed and organizing aneurysms) that developed prior to euthanasia and sampling and are more likely to be related to net cleaning. Thus, the unexpected finding of fewer fish with acute aneurysms at eight days after net cleaning could be related to differences during capture and euthanasia of the fish, although the same method and drug was used for euthanasia at all sampling points. In any case it is unlikely that net

cleaning should reduce the risk of acute aneurysms and haemorrhages. Recording vascular lesions classified as acute, subacute, and chronic separately, could be helpful to study the potential healing of lesions over time. However, no increase in median counts of subacute or chronic vascular lesions could be seen across the different time points (Table 3).

The biomass or species composition of the fouling communities present on the cage nets were not examined in the current study. A semiquantitative scoring of nets and net images give a rough estimate of level of fouling and the observation of the hydroid (*E. larynx*) by site staff and in a biofouling-sample confirms the presence of this organism at the site. However, the proportion and biomass of *E. larynx* present at time of washing and thus the concentration of potentially harmful hydroid fragments in the water during and after net cleaning is unknown. Previous research on biofouling communities in aquaculture in Norway, found *E. larynx* to be one of the dominant biofouling species (Bloecher, 2013; Bloecher et al., 2013; Bloecher et al., 2015; Bloecher et al., 2019; Guenther et al., 2009; Guenther et al., 2010; Napsøy, 2020) and the highest load of these organisms were found between August and November (Bloecher, 2013; Guenther et al., 2009). Hydroids have been shown to dominate the biofouling community in the early phase of net colonization (Bloecher et al., 2013; Boero, 1984) and hydroids are most abundant in South-west and Central Norway (Bloecher, 2013; Guenther et al., 2010). The time of year, phase of net colonization and geographical location would have favoured growth of hydroids on the project cages, but examination of the biofouling communities is necessary to confirm this.

The low number of pens included, and the lack of characterization and quantification of the net biofouling are important limitations of the current study. It is likely that differences in biofouling, biofouling strategies, site-specific factors, health status and size of the fish at time of net cleaning together will determine whether net cleaning negatively impacts gill health and if so the degree of gill damage and dysfunction. Studies have shown that the composition and biomass of fouling communities can vary with immersion time, season, between and within sites and as a result of different cleaning strategies (Bloecher et al., 2013; Bloecher et al., 2019; Carl et al., 2011; Guenther et al., 2009). Local factors such as positioning of cages, lice skirts, exposure to currents and waves, stage of tidal flow, as well as net cleaning device, cleaning efficiency and pressure are likely to affect to the concentration of potentially harmful particles in the water and how long fish are exposed to these (Bannister et al., 2019; Bloecher et al., 2019; Lewis and Metaxas, 1991). In addition, the health state of the fish and their exposure to other stressors may impact how they handle potential direct gill damage by biofouling debris, as stress and reduced health can lead to reduced capability to heal tissues (Christian et al., 2006; Sveen et al., 2018) and fight off potential secondary infections (Rojas et al., 2002). As such it possible that the results would have been different if more sites and pens were included the study, and more research is required to understand if and how different environmental factors, production practices and fish states impacts whether net cleaning has a negative effect on fish health. In addition, further studies are needed to understand the potential impact of the multiple net cleaning events that occurs during a regular production cycle (Bloecher and Floerl, 2020a). Access to this knowledge will be important to optimize biofouling management strategies in salmonid aquaculture.

Authorship statement

All persons who meet authorship criteria are listed as authors, and all authors certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript. The name of each author must appear at least once in each of the three categories below. Category 1 Conception and design of study: LØ, MS, MS, KIL; acquisition of data: AS, HR; analysis and/or interpretation of data: HR, LØ, MS, AN.

Category 2 Drafting the manuscript: LØ; revising the manuscript critically for important intellectual content: MA, KIL, MS, AN, HR, AS. Category 3 Approval of the version of the manuscript to be published (the names of all authors must be listed): MA, KIL, MS, AN, HR, AS, LØ.

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Supplementary file S1: Histology score system

The two-step score system is based on first estimating the number of lamella available for evaluation in each sample, and then using this estimate to calculate the estimated percent of gill tissue affected by different lesions.

Step 1. Estimate the number of lamellae in the sample.

In a small subset of fish (n=12, n=4 per pen) representative of the samples to be analyzed one filament is measured and the number of lamellae was counted. This filament should have adequate orientation (i.e., lamellae present on at least one side of the filament along the entire length of the filament). Then the number of lamellae per millimeter filament was calculated for each sample (#lamellae/filament length in millimeter) and the mean number lamella per mm filament across all samples was used in further calculations to estimate the number of lamella available for examination in each gill sample. The mean number of lamellae per millimeter filament was 35,4, with a range of 33,8-39,2.

For each sample to be scored the number of filaments was counted and a filament deemed representative of the mean filament length in the sample was measured. All filaments were then assessed for quality of plane of section and the number of filaments with good, average, and poor plane of section was counted. For a filament with good plane of section lamellae were present on both sides along the majority (> 80%) of the filament. In filaments with average plane of section lamellae were present on both sides along 50 to 80% of the filaments, while lamellae were present on both sides on less than 50% of the filament as poor plane of section. Based on the above information, the estimated number of lamellae available for assessment in each sample was calculated as follows:

1. Estimated number of secondary lamellae per filament = Length of average filament in millimeter * 35,4 (mean #lamella per mm filament)

2. Estimated number of secondary lamellae in the sample = (number of filaments in good plane of section * estimated number of secondary lamellae per filament) + (number of filaments in average plane of section * estimated number of secondary lamellae per filament *0.8) + (number of filaments in poor plane of section * estimated number of lamellae per filament *0.5)

Step 2. Estimating the % gill tissue affected by a given gill lesion.

For each of the categories of gill lesions that were recorded quantitatively and semi-quantitatively the number of lamellae affected by each lesion were counted. The percent of affected lamellae was subsequently calculated as:

Percent affected lamellae = (number of lamellae with a given lesion / estimated number of lamellae in the sample) *100

Counts and percent of gill hyperplasia, acute vascular lesions, subacute vascular lesions, chronic vascular lesions, and necrosis was recorded. These lesions were defined as follows:

Acute vascular lesions

  o Aneurysms, i.e., dilation of lamellar vascular sinusoids with rupture and loss of pillar cells, but without proliferation of lamellar epithelium, fibrin deposition or thrombosis of the affected sinusoid.

  o Haemorrhage, extravascular erythrocytes within the gill tissue. Red blood cells free between lamellae or filaments were not counted as haemorrhage.

Subacute vascular lesions

- o Aneurysms with fibrin deposition, but without proliferation of lamellar epithelium or infiltration of intact cells into the thrombosed vascular space.
- o Thrombi in lamellar sinusoids with normal diameter, but without proliferation of lamellar epithelium or infiltration of intact cells into the thrombosed vessel.

Chronic vascular lesions

- o Thrombosed aneurysms or thrombi with 1 or more of:
  - ▪ Proliferation of surrounding lamellar epithelium (at least 2 cell-layers thick)
  - ▪ Recanalization of thrombus
  - ▪ Infiltration of intact cells (presumed fibroblasts or endothelial cells) into the thrombosed vessel
  - ▪ Thickening of the vascular basal lamina
  - ▪ Infiltration of fibrovascular tissue with or without obliteration of the vessel lumen

Hyperplasia

- o Proliferation of lamellar epithelium cells to the extent that at least 80% of the interlamellar space is filled with hyperplastic cells. Lamellae on each side of the affected interlamellar spaces are counted.

Necrosis

- o Cell death of a least one lamella, including epithelium and pillar cells.

In addition, presence or absence of the following lesions or pathogens were recorded as 0 or 1:

- Epithelial cell necrosis or apoptosis

    o Lamellar epithelial cells with hypereosinophilic cytoplasm and/or nuclear pyknosis, karyorrhexis or karyolysis

    o Lamellar epithelial cells with margination or clumping of nuclear chromatin

- Adhesion of lamellae

    o Adhesions between one or more lamellae

- Hypertrophy/swelling of epithelial cells

    o Cuboidal or swollen gill epithelium lacking signs of necrosis or apoptosis – i.e. not hypereosinophilic cytoplasm or nuclear pyknosis, karyorrhexis or karyolysis

- Lamellar oedema/"lifting"

    o Accumulation of eosinophilic homogenous or granular material (presumed fluid) between the lamellar basal lamina and lamellar epithelial cells.

- Inflammation in the filaments

    o Infiltration of any type of presumed inflammatory cells in the filament.

- Subepithelial inflammation

    o Infiltration of any presumed inflammatory cells in the lamellae

Inflammation was also assessed by examination of 3 filaments at high magnification, counting 10 lamella per filament and recording how many of these were infiltrated with presumed inflammatory cells. Filaments with a good plane of section (>80% of length with lamellae on both sides) were selected. If no filaments with good planes of section were obvious for assessment, the filaments with average orientation (50 to 80% lamellae on both filament sides) were assessed instead. Percent of lamella with inflammation was then calculated as follows:

1. *Percent inflammation = (Number of lamellae with inflammation/number of lamellae examined) *100*

III **Effects of thermal and mechanical delousing on gill health of farmed Atlantic salmon (*Salmo salar* L.)**

Effects of thermal and mechanical delousing on gill health of farmed Atlantic salmon (*Salmo salar* L.)

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A B S T R A C T

Widespread resistance to medicinal delousing compounds has resulted in non-medicinal delousing methods being the preferred treatment option for sea lice infestation in Norwegian aquaculture. Thermal delousing involves submerging fish in water with a temperature of 28–34 °C for 20–30 s, while mechanical delousing entails removing the lice with flushing, brushing or turbulence. As mechanical and thermal delousing became increasingly common, injuries and increased mortality were often reported in association with treatments and currently mechanical injuries sustained during non-medicinal delousing are considered to be an important cause of mortality and reduced fish welfare. Gill injuries have been reported after non-medicinal delousing and it has been shown that increased temperatures can lead to differential gene expression in the gill tissue. It was therefore of interest to explore if thermal and mechanical delousing can compromise gill health. The objective of this study was to determine if thermal and mechanical delousing under a commercial setting can lead to gill damage, differential gene expression, and changes in pathogen prevalence and load in the gill tissue of farmed Atlantic salmon. To assess this, gill tissue from presumed healthy fish was collected prior to and at two time points post-thermal or mechanical delousing and examined using a detailed histopathological assessment protocol. Further, for gill tissue from the thermal delousing site, mRNA expression of markers of heat shock responses, hypoxia, inflammation, and repair were assessed by RT-qPCR analysis. RT-qPCR analysis for gill pathogens *Neoparamoeba perurans*, *Candidatus* Branchiomonas cysticola, *Desmozoon lepeophtherii* and salmon gill poxvirus (SGPV) was also conducted. Microscopic examination showed an increased percentage of gill tissue with vascular and hyperplastic lesions and an increase in the prevalence of putative gill pathogens observed post-treatment. Gene expression analysis revealed differential expression of genes involved in pathways of cell stress, inflammation, repair, and proliferation in the gill tissue after thermal delousing. Lastly, RT-qPCR analysis showed increased pathogen load of the putative gill pathogen *Ca.* B. cysticola after thermal delousing. The percentage of gill tissue affected was low, generally estimated to be <2%, and thus the clinical impact of these lesions remains to be established. However, the observed vascular and hyperplastic lesions will reduce overall gill capacity.

1. Introduction

Sea-lice is a big challenge for salmonid aquaculture (Costello, 2006) and control of this parasite represents a major cost for the industry (Abolofia et al., 2017; Iversen et al., 2017). Widespread resistance to medicinal delousing compounds has resulted in non-medicinal

delousing methods being the preferred treatment option in Norwegian aquaculture (Myhre Jensen et al., 2020; Overton et al., 2018; Sommerset et al., 2021; Sviland Walde et al., 2021; Aaen et al., 2015). Currently, two systems for thermal delousing and three different systems for mechanical delousing are commercially available in Norway. In addition, a non-medicinal combination treatment, involving thermal delousing

followed by flushing has recently been tested (Salvesen et al., 2021) and freshwater bath treatments has increasingly been used in recent years (Sommerset et al., 2021).

Thermal delousing involves submerging fish in a chamber with water with a temperature of 28–34 °C for 20–30 s (Holan et al., 2017; Noble et al., 2018). The sudden increase in water temperature causes the lice to detach from the fish and lice are then removed by filtration of the treatment water (Grøntvedt et al., 2015; Roth, 2016). Mechanical delousing involves flushing with water jets, flushing and brushing, or negative pressure and turbulence combined with flushing. All these delousing operations also entail crowding, pumping, and straining that may lead to stress, risk of hypoxia and mechanical injuries (Erikson et al., 2018; Gismervik et al., 2016; Gismervik et al., 2017; Grøntvedt et al., 2015; Oppedal et al., 2011; Roth, 2016; Skjervold et al., 2001).

Treatment effect and welfare impact of thermal and mechanical delousing systems have been evaluated by independent research institutions during the development of these systems (Erikson et al., 2018; Gismervik et al., 2017; Grøntvedt et al., 2015; Nilsen et al., 2010; Roth, 2016). These studies concluded that fish welfare and treatment effect of thermal delousing was acceptable (Grøntvedt et al., 2015; Roth, 2016), while effects on welfare were somewhat variable for mechanical delousing (Erikson et al., 2018; Gismervik et al., 2017; Grøntvedt and Kristensen, 2018; Nilsen et al., 2010). As mechanical and thermal delousing became increasingly common, cases of injuries and increased mortality were reported in association with treatments and currently mechanical injuries sustained during non-medicinal delousing is considered to be an important cause of mortality and reduced fish welfare in Norwegian aquaculture (Hjeltnes et al., 2018; Overton et al., 2018; Sommerset et al., 2021; Sviland Walde et al., 2021).

Gill injuries described after thermal and/or mechanical delousing include gill aneurysms, hemorrhages, thrombi, and unspecified injuries (Gismervik et al., 2017; Gismervik et al., 2019; Hjeltnes et al., 2018; Jørgensen and Rød, 2019; Poppe et al., 2018; Sommerset et al., 2021). Mechanical injuries occurring during delousing may initiate inflammatory and regenerative/reparative responses in affected tissues associated with differential expression of genes involved in these processes. Experimental exposure of fish to water with elevated temperatures can cause stress responses (Nakano et al., 2014), behavioural responses indicative of pain (Nilsson et al., 2019) and differential expression of genes encoding for, or increased levels of, heat shock proteins, antioxidant enzymes like superoxide dismutase, glutathione peroxidase and proteins of the ubiquitin-proteasome pathway (Ackerman et al., 2000; Basu et al., 2001; Buckley et al., 2006; Fangue et al., 2006; Nakano et al., 2014; Rebl et al., 2013). The stress response associated with crowding and handling during non-medicinal delousing may impact fish health and physiology and could potentially lead to reduced immune function and increased susceptibility to infectious disease (Espelid et al., 1996; Iversen et al., 2005; Nardocci et al., 2014).

Gill disease is an important cause of reduced welfare, mortality, and increased production cost in salmon farming (Shinn et al., 2015; Sommerset et al., 2021). It is of interest to explore if thermal and mechanical delousing can compromise gill health as gill injuries have been reported after non-medicinal delousing and increased temperature by itself can lead to differential gene expression in the gill tissue (Fangue et al., 2006; Rebl et al., 2013). The objective of this study was to determine if thermal and mechanical delousing under a commercial setting can lead to gill damage, differential gene expression in the gills, and changes in pathogen prevalence and load in the gill tissue of farmed Atlantic salmon. To assess this, gill tissue from presumed healthy fish was collected prior to and at two time points post thermal or mechanical delousing and examined using a detailed histopathological assessment protocol. Furthermore, for gill tissue from the thermal delousing site, mRNA expression of markers of heat shock responses, hypoxia, inflammation, and repair were assessed by RT-qPCR analysis. RT-qPCR analysis for gill pathogens *Neoparamoeba perurans*, *Candidatus* Branchiomonas cysticola, *Desmozoon lepeophtherii* and salmon gill poxvirus (SGPV) was also

conducted. More knowledge about the potential role of non-medicinal delousing methods in development of gill disease and gill injury will assist fish farmers and fish health personnel in making informed decisions about delousing strategies.

2. Material and methods

2.1. Study populations

2.1.1. Thermal lice treatment

The site was located in West Norway in Vestland county and consisted of eight pens, seven of which contained Atlantic salmon (*Salmo salar* L.). Of these, three pens were included in the study. The pens and site were selected based on the following criteria: a) the fish had not previously been deloused with thermal or mechanical methods, b) there were no known gill health issues, issues related to plankton or jellyfish or preferably other diseases at the site, c) fish were comparable regarding time of sea transfer, strain (etc.). The fish at the site originated from two different hatcheries and were sea transferred in November 2018. There were no known infectious or non-infectious diseases at the site. Mean fish weight, fish number and biomass per pen on the treatment day are summarized in Table 1. One month before treatment sea temperature ranged from 12.8 to 15.7 °C and oxygen saturation ranged from 59 to 87%.

Delousing was performed in September 2019 using a well-boat equipped with two treatment lines each with one Thermolicer unit (ScaleAQ AS). Fish were crowded and pumped using an injector pump with a negative pressure of 0.32 bar, passed over a grid to remove seawater and then entered the treatment loop containing heated water. After treatment the fish passed a second grid to remove treatment water and detached lice, before being transferred back to the pen. The treatment water was recirculated and heated, filtered and oxygenated during delousing. Crowding time ranged from 51 to 60 min, and time from start to end of treatment ranged from 2 h15 minutes to 3 h per pen. The treatment water temperature was 33.9 °C and time in the treatment loop was 28 s, while the sea water temperature was 15.5 °C. No adverse events were reported during the treatment.

2.1.2. Mechanical delousing

The sea site was located in Northwest Norway in Trøndelag county and consisted of eight pens with Atlantic salmon. The same selection criteria as used for thermal delousing was applied. Fish were sea transferred in September 2019. Heart and skeletal muscle inflammation (HSMI) had been diagnosed in pen E and two other pens at the site not included in the study 14 days prior to delousing. The risk associated with handling and treatment was evaluated as low by the attending veterinarian. Sea temperature ranged from 8.53 to 11.91 °C one month before treatment, oxygen saturation ranged from 89.12 to 117.65% and salinity ranged from 30.75 to 32.54‰. Mean fish weight, fish number and biomass per pen on the treatment day are summarized in Table 1.

The three study pens were deloused July 2020 and treatment was performed using a vessel containing three FLS delousing lines delivered by Flatsetsund Engineering in 16.12.2017 (FLS-system no. 3). The system had not been modified since delivery. During FLS delousing fish were crowded, pumped into a pipe and through a treatment unit

Table 1
Mean fish weight (kg), fish count and biomass (kg) for each pen at the day of delousing.

Site	Pen	Mean weight (kg)	Fish count	Biomass (kg)
Thermal delousing	A	2.75	168,048	433,477
	B	1.9	149,165	283,414
	C	2	145,767	291,534
Mechanical delousing	D	3.5	42,289	149,135
	E	3.0	98,520	294,692
	F	3.1	64,871	199,689

containing water jets flushing the lice from the skin surface of the treated fish, before the fish passed over a grid and back into the pens. Fish would generally enter the pipe backwards and were pumped from the pen and through the closed system using negative pressure generated by an ejector pump (PG flow solutions AS). Crowding time prior to treatment was approximately 1 h and total time used from start of crowding to end of treatment, for a pen, ranged from 4.2 to 6.28 h. Flushing pressure of the water jets was 0.76 to 0.77 bar, and the FLS unit used contained two water jets oriented perpendicular (90°) to the direction of fish movement and pipes. According to the manufacturer the estimated treatment time per fish is 2 s, while time through the entire system is 10–15 s per fish. Sea temperature ranged from 8.7 to 12 °C and oxygen levels never dropped below 89.5% during crowding. No adverse events were reported during the treatment.

2.2. Sampling

Thirty fish were sampled per pen and timepoint ($n = 270$ fish for mechanical delousing and $n = 269$ fish for thermal delousing). Due to practical concerns pens were sampled 1 to 2 days prior to treatment (time point 0) and 1 or 2 days after both thermal and mechanical delousing (time point 1). The second sampling post-treatment was conducted at day 6 or 7 after mechanical delousing and at 8 to 9 days after thermal delousing (time point 2). On each sampling occasion fish were crowded using feeding and a purse seine, and 30 fish were selected from the seine with a dip net, as described in the standard operating procedure of the fish farms. The aim was to sample up to 15 fish with clinical signs of disease and 15 healthy fish per pen after delousing. However, no moribund fish or fish with overt signs of gill disease were observed, except for two moribund fish sampled from pen F at six days after mechanical delousing. Euthanasia was achieved with an overdose of benzocaine (Benzoak vet, ACD Pharmaceuticals AS). Within five
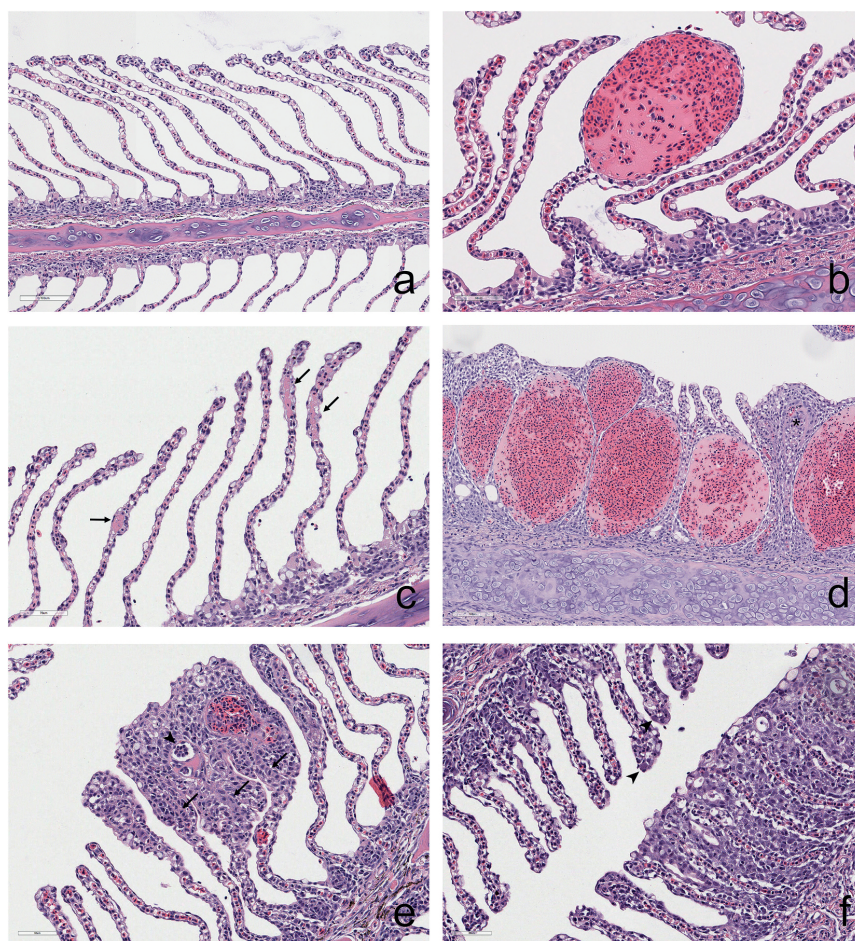
Fig. 1. Normal gill tissue and histology lesions recorded as counts and percent. All tissues stained with haematoxylin and eosin. a) Normal gill tissue. b) Acute aneurysm. c) Thrombi (arrows). d) Reactive and reparative vascular lesions – aneurysms with associated lamellar epithelial hyperplasia and fusion. Also note vascular lesion with debris and infiltration of cells into the former vascular lumen (*), likely a resolving thrombus formed earlier from an aneurysm. e) Reactive and reparative vascular lesions – thrombosed aneurysms with lamellar epithelial hyperplasia and presumed recanalization (arrowhead), loss of normal vascular lumina (arrows) replaced by intact cells. f) Lamellar epithelial hyperplasia. Also note intracellular bacteria, epitheliocysts, (arrowheads).

minutes after euthanasia the second left gill arch was sampled for histology, while tissue samples from the third left gill arch was placed in RNAlater (Sigma-Aldrich) for qPCR and gene expression analysis. An overview of the number of samples submitted for different types of analysis in connection with thermal delousing is available in Supplementary material 1, Table S1.

2.3. Treatment related mortality

Recorded daily mortality counts and percent per pen were provided by the farming companies. Mortality rates per pen within 7 days before delousing, and within 1, 7 and 14 days after delousing and the second week after treatment was calculated as follows (Toft et al., 2004):

$$\text{Mrate} = \frac{\text{number of dead fish during time period}}{\frac{\text{number of fish at risk at start} + \text{number of fish at risk at end}}{2}} \text{x time period}$$

Delta mortality rates were calculated by subtracting the mortality rate before treatment from the mortality rate after treatment as reported by Sviland Walde et al., (2021):

$$\Delta\text{Mrate} = \text{Mrate\_post treatment} - \text{Mrate\_prior to treatment}$$

2.4. Histopathology

Gills were fixed in 10% buffered formalin (4% formaldehyde, 0.08 M sodium phosphate, pH 7.0), processed routinely, and sections were stained with haematoxylin-eosin (HE) and scanned for histopathologic examination as previously described in Østevik et al., (2021). Slides were randomized using computer generated random numbers, and the pathologist was "blinded" regarding pen, time point or results of qPCR-

analysis or gene expression analysis if available. A two-step assessment system developed by the authors (Østevik et al., 2021) was used (for details see Supplementary material 2). Briefly, first the number of lamella available for evaluation in each sample was estimated, and then all affected lamella with hyperplasia, necrosis, thrombi (subacute vascular lesions), acute and/or reactive and reparative (chronic) vascular lesions were counted (Fig. 1). These counts were used to calculate the estimated percent of gill tissue affected for each type of lesion.-----

In addition, presence, or absence of the following lesions (Fig. 2) and any pathogens and possible pathogen-associated lesions (Supplementary material 3, Fig. S1) present were recorded as 0 or 1 (dichotomous variables):

- Epithelial cell necrosis
- Adhesion of lamella
- Hypertrophy/swelling of epithelial cells
- Lamellar oedema/"lifting"
- Inflammation of the filaments
- Subepithelial lamellar inflammation

In contrast to the earlier publication (Østevik et al., 2021), subepithelial inflammation was defined as infiltration of any presumed inflammatory cells together with presence of pale, yellow to brown, intracellular, granular pigment in the lamellae.

2.5. RT-qPCR for gill pathogens

A subset of samples collected during thermal delousing were examined with RT-qPCR for the most commonly detected gill pathogens in
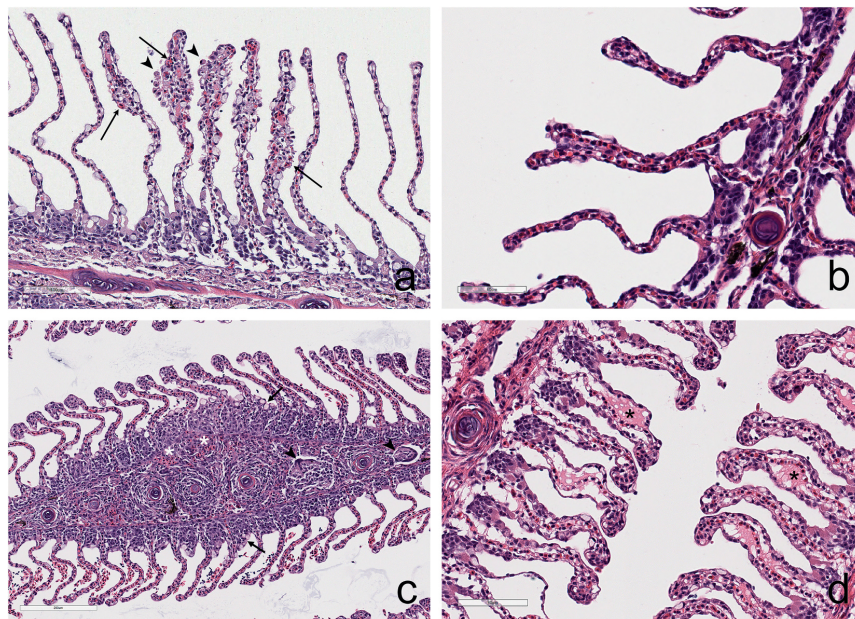
Fig. 2. Histology lesions recorded as dichotomous variables. All tissues stained with haematoxylin and eosin. a) Epithelial cell necrosis (arrowheads), also note lamellar thrombosis and hemorrhage (arrows). b) Adhesions between lamellae. c) Inflammation of the filament – expansion of the filament due to an inflammatory infiltrate consisting of mononuclear cells and multinucleated giant cells (arrowheads). Also note hemorrhage (asterisk) in the filament and increased number of goblet cells in the overlying interlamellar epithelium (arrows). d) Lamellar oedema/"lifting" – eosinophilic material presumed to be proteinaceous fluid (*) beneath the lamellar epithelium.

Norwegian salmonid mariculture; *Candidatus* Branchiomonas cysticola ($n = 198$), *Desmozoon lepeophtherii* (syn. *Paranucleospora theridion)* (n = 198), salmon gill poxvirus (SGPV) ($n = 110$) and *Neoparamoeba perurans* ($n = 110$). Samples for RT-qPCR-analysis was selected independently of the histology results with exception of 20 samples from pen B ($n = 10$) and C ($n = 10$) selected based on low (n = 10) or high (n = 10) percentage of vascular lesions.

For nucleic acid isolation gill tissue were homogenized in a tube containing 650 µl MagNa pure 96 RNA tissue lysis buffer and 2 steel beads, using a FastPrep96 (Biomedical). The gills were homogenized twice for 1 min at 1600 rpm, with 2 min of cooling at 4 °C in between. Nucleic acids (DNA and RNA) were extracted from 500 µl of the homogenized gill tissue using Roche's MagNA Pure 96 instrument, using the MagNA Pure 96DNA and Viral NA large volume kit, and the protocol Pathogen Universal 500. Extracted DNA and RNA were eluted in 50 µl final volume of the supplied kit elution buffer. 2.5 µl of extracted DNA and RNA was used in a duplex reaction for each pathogen together with an assay for salmonid elongation factor 1 alpha (EF1α), using Roche's Light Cycler Multiplex RNA Virus Master, in a total volume of 10 µl in each well. Primer and probe concentrations were 500 µM and 200 µM, respectively, for all pathogen assays, and 130 µM and 100 µM for the EF1α-assay. Samples were run in a single well for each sample and for each pathogen assay. EF1α was used as an internal control. A notemplate (water control) was included to detect potential contamination of reagents with genetic material. The primers and probes used are listed in Supplementary material 1, Table S2. The efficiency of each assay was calculated by analyzing a 10-fold dilution series in triplicates (Supplementary Table S3). The RT-qPCR was performed on Roche's LightCycler 480 II, using the following cycling profile: Reverse transcription: 50 °C for 10 min. Initial denaturation: 95 °C for 30 s. Amplification, 45 cycles: 95 °C for 5 s, 60 °C for 30 s. Cooling: 40 °C for 30 s.

Normalized Ct-values were calculated for pathogens *Ca.* B. cysticola and *D. lepeophtherii* using the following formula:

$$\text{Normalized Ct} = \frac{(\text{Efficiency EF1}\alpha \text{ assay}^{\hat{C}t \text{ EF1}\alpha})}{(\text{Efficiency pathogen assay}^{\hat{C}t \text{ pathogen}})}$$

Normalized fold change was then calculated using the mean of the normalized Ct-values of samples collected pre-delousing for each pen:

$$\text{Normalized fold change} = \frac{\text{Normalized Ct}}{(\text{mean Normalized Ct for pen } A - C)}$$

2.6. Gene expression analysis

A subset of samples was submitted for qPCR-analysis of 10 genes to study the responses to thermal delousing. These included samples from 142 fish, 13 to 18 per pen and time point, which were also analyzed for *Ca.* Branchiomonas cysticola and *D. lepeophtherii* RT-qPCR. In addition, 5 to 18 of these gills was analyzed for *N.perurans* and SGPV. Samples for analysis were selected independently of the histology results. Genes involved in response to heat exposure, hypoxia, regulation of inflammation and proliferation/repair were selected for gene expression analysis. The genes and primers are listed in Table 2.

Nucleic acids were isolated and extracted as described in section 2.5 above. RT-qPCR was performed using QuantiFast SYBR Green RT-PCR Kit (Qiagen) and the AriaMx Real-Time PCR System (Agilent). For each gene, 100 ng total RNA was used as a template in a mixture of specific primers (10 µM) and Master Mix in a final volume of 25 µl following manufacturer's instruction. The mixtures were first incubated for reverse transcription at 50 °C for 10 min and subsequently for PCR initial activation at 95 °C for 5 min, followed by 40 amplification cycles (10 s at 95 °C and 30 s at 60 °C). The specificity of the PCR products from each primer pair was confirmed by melting-curve analysis and agarose gel electrophoresis. The efficiency of each primer pair was determined by the standard curve method (Supplementary material 1,Table S4), and the Pfaffl method (Bustin et al., 2005) was subsequently used for quantification of cycle threshold values using β-actin as the housekeeping gene.

2.7. Statistical analysis

Statistical analysis of results was undertaken in STATA (StataCorp. 2015. Stata Statistical Software: Release 14. College Station, Texas, United States). Box and whisker plots were produced to provide a visual presentation of fold change of normalized *Ca.* B. cysticola and *Desmozoon lepeophtherii* Ct-values, $2^{-\Delta\Delta \text{ Ct values}}$ of selected genes and percent gill lamella with hyperplasia, thrombi, acute and reactive and repairing vascular lesions per pen and time point. In both the histopathology datasets the variance of the lesion counts was considerably greater than the mean and there were not an excessive number of samples with zero counts. Thus, to determine if there was as significant change in the number of gill lesions before and after delousing, we used a series of negative binomial regression models with lesion counts as the response

Table 2
Genes, pathways, and primers used in qPCR and gene expression analysis.

Pathways	Gene	Sequence	Accession no.
Housekeeping gene	β-actin	Bactin-F CCAGTCCTGCTCACTGAGGC Bactin-R GGTCTCAAACATGATCTGGGTCA	AF012125
Cellular stress, chaperone protein	Heat shock protein 70 (HSP70)	HSP70-F CCCCTGTCCCTGGGTATTG HSP70-R CACCAGGCTGGTTGTCTGAGT	XM_014197598
Response to hypoxia, transcription factor	Hypoxia-inducible factor-1α (HIF-1α)	HIF1A-F CCACCTCATGAAGACCCATCA HIF1A-R TCTCCACCCACACAAAGCCT	XM_014189950
Pro-inflammatory cytokine, Th1 response	Interleukin-1β (IL-1β)	IL1b-F TGAAGTCCATCAGCCAGCAG IL1b-R GGATGGTGAAGGTGGTGAGG	XM_014170479
Pro-inflammatory cytokine, Th1 response	Tumor necrosis factor-α (TNF-α)	TNFa-F ATGGAAGACTGGCAACGATG TNFa-R TCACCCTCTAAATGGATGGC	NM_001123589
Pro-inflammatory cytokine, Th1 response	Interferon-γ (IFN-γ)	IFNg-F AAGGGCTGTGATGTGTTTCTG IFNg-R TGTACTGAGCGGCATTACTCC	NM_001171804
Anti-inflammatory cytokine	Interleukin-10 (IL-10)	IL10-F GGGTGTCACGCTATGGACAG IL10-R TGTTTCCGATGGAGTCGATG	XM_014168417
Growth factor, suppress acute inflammation	Transforming growth factor β (TGF-β)	TGFb-F GCCATCCGTGGACAGATACT TGFb-R TCTCCCTCCTGGTCAATCTCT	XM_014129261
Pro-inflammatory cytokine, Th2 response	Interleukin-4/13α (IL-4/13α)	IL4/13a-F GCATCGTTGTGAAGAGCCAAGA IL4/13a-R GAAGTCTCCTCAGCTCCACCT	NM_001204895
Pro-inflammatory cytokine, Th2 response	Interleukin-4/13β2 (IL-4/13β2)	IL4/13b2-F GTGAAGGAGAACGGTGATGAGGAACAGC IL4/13b2-R GGCACAGTTGAAGAGGTTTGTCAGGAGAT	HG794525
Growth factor receptor, epithelial proliferation, and repair	Epidermal growth factor receptor (EGFR)	EGFR-F GACACCAAGTTCTACCAGAGCCTAATCAGT EGFR-R GCGTCCACAGCGTCCTCCAT	XM_014191766

variable and sampling time and cage as predictor variables. Filament count was added as the exposure to correct for variation in the number of filaments available for evaluation in the tissue samples. The incidence rate ratio (IRR) was calculated for the two time points after delousing which shows the factor difference for the number of lesions found at the two time points after delousing compared to before delousing. To determine if there was as significant change in the number of fish with gill lesions or microorganisms recorded as dichotomous variables, we used a series of logistic regression models with each category of lesion as the response variable and sampling point and cage as predictor variables. The odds ratio (OR) was calculated for the two time points after delousing, using the pre-delousing observation as a baseline.

Relative expression of the selected genes were calculated using the ΔΔCT method (Livak and Schmittgen, 2001) using β-actin as a reference gene. Fold change of normalized *Ca.* B. cysticola Ct-values and $2^{-\Delta\Delta Ct}$ values were log transformed or square root transformed to achieve approximate normal distribution. To assess if there was a significant difference in fold change before and after thermal delousing, we used a series of linear regression models with transformed fold values as response variable and sampling point and cage as predictor variables. Post-estimation was performed by visual assessment of qq-plots. A series of Kruskal-Wallis tests was used to assess possible differences between time points for fold change of normalized *D. lepeophtherii* Ct-values and fold change of gene expression when criteria for normality were not met. For all analyses differences were considered significant at a probability level of $p < 0,05$. Possible association between percentage of vascular and hyperplastic lesions and fold change in gene expression and normalized Ct-values for *D. lepeophtherii* and *Ca.* B. cysticola were assessed by producing scatter plots and computation of Pearson's correlation coefficient.

3. Results

3.1. Treatment related mortality

The percent daily mortality increased during and after delousing for all pens, but there was considerable variation in mortality rates between pens for both thermal and mechanical delousing (Fig. 3a and b). Mortality rates increased following delousing for all pens (Supplementary material 1, Table S5), although there was a drop in mortality at day one after mechanical delousing for pen E. The delta mortality rate at one day after treatment was generally higher than the delta mortality rate at 1 week after treatment, indicating that most of the mortality occurred during or a short time after delousing. However, the percent daily

mortality remained elevated for more than a week after both thermal and mechanical delousing before approaching the levels observed before treatment.

3.2. Histopathology

A low percentage of sum vascular lesions (median < 0.6%) and lamellar epithelial hyperplasia (median < 0.1%) were observed in the gills before thermal and mechanical delousing. After delousing, there was an increased percentage of hyperplasia and sum vascular lesions (relative to pre-delousing) for both treatments (Fig. 4a-d). The results of statistical analysis of histopathology data are found in Tables 3 to 6 and are summarized below. The number and prevalence of fish with different lesions and pathogens and mean, median, minimum, and maximum lesion counts per sampling point are found in Supplementary material 1,Tables S6 and S7.

3.2.1. Thermal delousing

At 8 and 9 days after thermal delousing, there was a significant increase in the number of thrombi and reactive and repairing vascular lesions, while there was no significant difference in the number of acute vascular lesions (hemorrhages and acute aneurysms) between the different time points (Fig. 4c, Table 3). The number of lamellae with hyperplasia was increased at 1 and 2 days and 8 and 9 days after delousing (Fig. 4a, Table 3). Thrombi were largely detected in normal diameter lamellar sinusoids (Fig. 1c) and appeared to be randomly distributed in the gill tissue. Inflammation of the filament and/or inflammatory cells infiltrating the lamellar epithelium and occasionally epitheliocysts were observed in hyperplastic lesions. The most severe hyperplastic lesions were in the filament tips and were frequently found concurrently with moderate to marked inflammation of the filament and reactive and reparative vascular lesions of the lamella. Focal necrosis of lamella and underlying tissue were found in inflamed filament tips in three fish.

The prevalence of lamellar adhesion increased at 8 and 9 days after treatment, while the prevalence of lamellar oedema was decreased at 1 and 2 days post delousing (Table 4). However, both lamellar oedema and adhesion were observed in relatively few gills (Supplementary material 1,Table S7). Epithelial cell necrosis or filament inflammation did not differ across timepoints. The prevalence of epitheliocysts and subepithelial inflammation increased at both time points after delousing, and there was a significantly increased prevalence of fish with *Trichodina* spp. at 8 and 9 days after delousing. Epitheliocysts were found in 239 fish, while subepithelial inflammation was found in 105
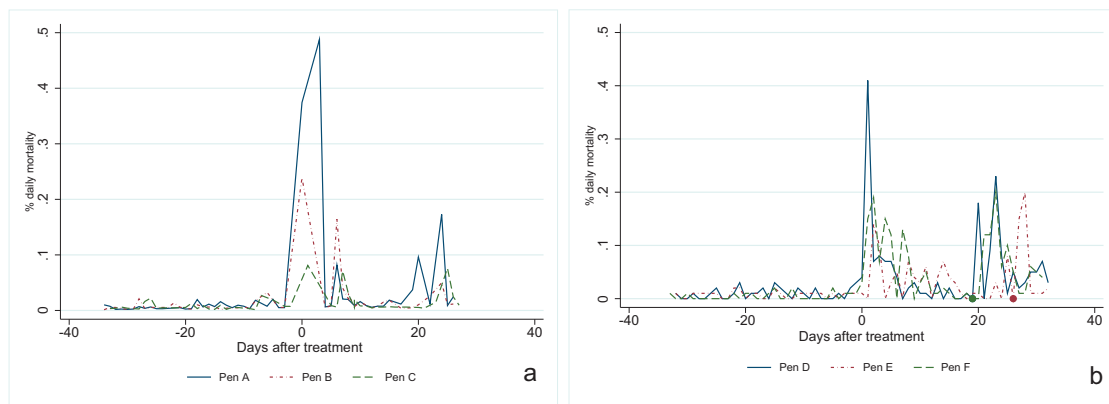

Fig. 3. Percent daily mortality per pen the month before and after the first delousing and sampling for a) thermal delousing and b) mechanical delousing. A new subsequent delousing event is represented by circles and involved all three pens, with treatment occurring at the same day for pen D and F.
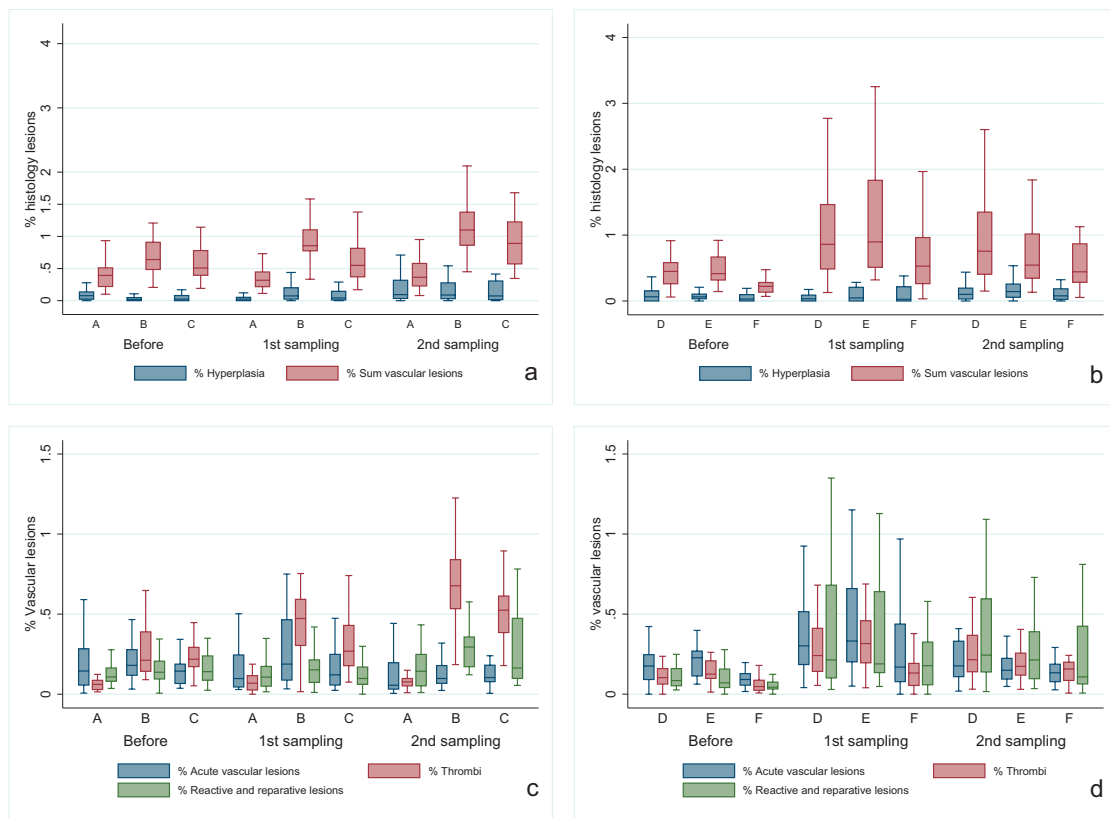
Fig. 4. Percentage gill tissue with lesions recorded by microscopic examination. Box and whisker plots show percentage gill tissue with hyperplastic and vascular lesions per pen and timepoint ($n = 29$–30) for a) thermal and b) mechanical delousing. Percentage of gill tissue with the different categories of vascular lesions are shown for c) thermal and d) mechanical delousing. Outliers are excluded for clarity.

fish. Amoeba associated with segmental hyperplasia was found in 2 fish but was not observed in the 4 fish with positive *N.perurans* qPCR-results.

3.2.2. Mechanical delousing

There was a significant increase in the number of all categories of vascular lesions at 1 and 2 days after mechanical delousing, and significantly higher number of thrombi and reactive and reparative vascular lesions at 6 and 7 days after treatment (Fig. 4d, Table 5). The number of lamellae with hyperplasia increased at both 1 and 2 days after and at 6 and 7 days after delousing (Fig. 4b, Table 5). Vascular lesions primarily consisted of aneurysms, with relatively few thrombi identified in normal diameter lamellar sinusoids. Hyperplasia was most often, but not exclusively, observed in areas with vascular lesions. The most severe hyperplastic and vascular lesions were located in filament tips, while the middle and basal aspect of filaments were less affected. Focal necrosis of lamella and underlying tissue was found in 3 fish.

There was a decreased prevalence of epithelial cell necrosis, lamellar adhesion, and inflammation of the filament at 1 and 2 days post delousing compared to before delousing, however, this was not evident at 6 and 7 days after delousing (Table 6). Prevalence of epitheliocysts and lamellar oedema did not differ between time points, but there was a slight and significant increase in the prevalence with subepithelial inflammation and *Ichthyobodo* spp. parasites at 6 and 7 days compared to before delousing. Segmental hyperplasia suggestive of amoebic gill

disease was observed in three fish, but amoeba was not observed in these gills or any other samples. Epitheliocysts were found in 230 of 270 samples, most often without any associated inflammation. Subepithelial inflammation were found in 55 fish and parasites consistent with *Ichthyobodo* spp. were found in 18 fish.

3.3. RT-qPCR

RT-qPCR analysis was only performed on a subset of samples collected in association with thermal delousing. *N. perurans* was found in 4 of the tested samples ($n = 110$), all collected 8 to 9 days post delousing, and from pens A and C. SGPV positive samples were found in 3 of those tested ($n = 110$), 1 positive per sampling time. *Ca.* B. cysticola and *D. lepeophtherii* was found in 100% and 98.99% of samples tested ($n = 198$), respectively. There was a statistically significant increase in the pathogen load of *Ca.* B. cysticola at both time points after thermal delousing (Fig. 5a, Table 7), while there was no significant difference in pathogen load for *D. lepeophtherii* (Fig. 5b, data not shown).

3.4. Gene expression analysis

The gene expression analysis included 13 to 18 fish per pen and time point ($n = 142$). Most genes showed up-regulation at 1 and 2 days post thermal delousing, returning towards pre-treatment levels by 8 and 9

Table 3

Thermal delousing. Results of statistical analysis for lesions recorded as counts. The negative binomial regression model for each lesion category includes sampling point ($n = 89$–90) and fish group/pen ($n = 89$–90, total $n = 269$).

Gill lesions	Time	Incidence rate ratio	P > |z|	95% Conf. Interval]
Acute vascular lesions	Before			
	1st sampling	0.98	0.889	(0.74, 1.30)
	2nd sampling	1.00	0.988	(0.75, 1.32)
Thrombi	Before			
	1st sampling	1.19	0.065	(0.99, 1.43)
	2nd sampling	1.95	0.000	(1.63, 2.34)
Reactive and repairing vascular lesions	Before			
	1st sampling	1.01	0.921	(0.79, 1.30)
	2nd sampling	2.19	0.000	(1.71, 2.81)
Sum vascular lesions	Before			
	1st sampling	1.07	0.438	(0.90, 1.28)
	2nd sampling	1.70	0.000	(1.43, 2.03)
Hyperplasia	Before			
	1st sampling	2.30	0.001	(1.40, 3.79)
	2nd sampling	4.09	0.000	(2.53, 6.62)

Table 4

Thermal delousing. Results of statistical analysis for gill lesions and pathogens recorded as dichotomous variables. The logistic regression model for each variable includes sampling point (n = 89–90) and fish group/pen (n = 89–90, total n = 269).

Gill lesions /Pathogens	Time	Odds ratio	P > |z|	95% Conf. Interval]
Epithelial cell necrosis	Before			
	1st sampling	0.71	0.255	(0.39, 1.28)
	2nd sampling	1.26	0.445	(0.69, 2.31)
Lamellar adhesion	Before			
	1st sampling	1.78	0.286	(0.62, 5.14)
	2nd sampling	6.72	0.000	(2.63, 17.23)
Lamellar oedema	Before			
	1st sampling	0.18	0.003	(0.06, 0.57)
	2nd sampling	1.07	0.852	(0.52, 2.23)
Inflammation filament	Before			
	1st sampling	0.78	0.415	(0.43, 1.41)
	2nd sampling	0.73	0.293	(0.40, 1.32)
Subepithelial inflammation	Before			
	1st sampling	1.85	0.079	(0.93, 3.68)
	2nd sampling	6.77	0.000	(3.44, 13.30)
Epitheliocysts	Before			
	1st sampling	2.24	0.067	(0.94, 5.32)
	2nd sampling	7.33	0.002	(2.07, 25.94)
Trichodina spp.	Before			
	1st sampling	1.77	0.061	(0.98, 3.20)
	2nd sampling	2.38	0.005	(1.30, 4.33)

Table 5

Mechanical delousing. Results of statistical analysis for lesions recorded as counts. The negative binomial regression model for each lesion category includes sampling point ($n = 90$) and fish group/pen ($n = 90$, total $n = 270$).

Gill lesions	Time	Incidence rate ratio	P > |z|	95% Conf. Interval
Acute vascular lesions	Before			
	1st sampling	3.15	0.000	(2.40, 4.13)
	2nd sampling	1.13	0.363	(0.86, 1.49)
Thrombi	Before			
	1st sampling	3.04	0.000	(2.42, 3.83)
	2nd sampling	2.18	0.000	(1.73, 2.74)
Reactive and repairing vascular lesions	Before			
	1st sampling	5.58	0.000	(4.10, 7.58)
	2nd sampling	4.38	0.000	(3.22, 5.95)
Sum vascular lesions	Before			
	1st sampling	3.70	0.000	(2.92, 4.68)
	2nd sampling	2.20	0.000	(1.73, 2.78)
Hyperplasia	Before			
	1st sampling	3.12	0.000	(1.92, 5.06)
	2nd sampling	3.23	0.000	(1.98, 5.26)

Table 6

Mechanical delousing. Results of statistical analysis for lesions and pathogens recorded as dichotomous variables. The logistic regression model for each variable includes sampling point (n = 90) and fish group/pen (n = 90, total n = 270).

Gill lesions /Pathogens	Time	Odds ratio	P > |z|	95% Conf. Interval
Epithelial cell necrosis	Before			
	1st sampling	0.38	0.016	(0.17, 0.84)
	2nd sampling	1.06	0.867	(0.55, 2.04)
Lamellar adhesion	Before			
	1st sampling	0.21	0.008	(0.07, 0.66)
	2nd sampling	0.64	0.293	(0.27, 1.48)
Inflammation filament	Before			
	1st sampling	0.28	0.000	(0.15, 0.52)
	2nd sampling	1.27	0.494	(0.65, 2.48)
Subepithelial inflammation	Before			
	1st sampling	1.38	0.424	(0.63, 3.05)
	2nd sampling	2.29	0.030	(1.08, 4.84)
Epitheliocysts	Before			
	1st sampling	2.0	0.129	(0.82, 4.87)
	2nd sampling	1.09	0.840	(0.49, 2.40)
Ichthyobodo spp.	Before			
	1st sampling	3.15	0.167	(0.62, 16.08)
	2nd sampling	5.53	0.031	(1.17, 26.07)

days after delousing (Fig. 6, Table 7 and Table 8). This included HSP70, TNF-α, IL-1β, TGF-β, and IL-4/13α. IL-10 and IFN-γ were upregulated at 1 and 2 days but were downregulated at 8 and 9 days post treatment. HIF-α and IL-4/13β2 were downregulated at 1 and 2 days and at both time-points post delousing, respectively. EGFR were upregulated at 1 and 2 days after treatment only in fish from pen A and C. Expression patterns of individual genes are shown in Fig. 6 and results of the statistical analysis found in Table 7 and Table 8. $2^{-\Delta\Delta \ Ct \ values}$ per pen and time point are summarized in Supplementary material 1, Table S8. Overall, the magnitude of the measured gene expression responses was small.

3.5. Correlation between histology lesions, qPCR-results, and gene expression fold changes

No clear patterns were observed when reviewing scatter plots (data not shown), and the Pearson's correlation coefficient between hyperplastic and vascular gill lesions and fold gene expression, and between gill lesions and Ct-values for *Ca.* B. cysticola and *D. lepeophtherii,* were low ($r^2 < 0{,}20$). Similarly, no clear pattern and a low correlation coefficient ($r^2 < 0{,}20$) was found for fold change in gene expression and normalized Ct-values for *Ca.* B. cysticola and *D. lepeophtherii*.

4. Discussion

The results suggest that thermal and mechanical delousing can have a negative impact on gill health. This is supported by the results of histology and gene expression analysis. Microscopic examination showed an increased percentage of vascular and hyperplastic lesions and an increase in the number of fish with putative gill pathogens observed after delousing. Gene expression analysis revealed differential expression of genes involved in pathways of cell stress, inflammation, repair, and proliferation in the gill tissue after thermal delousing. Lastly, RT-qPCR analysis showed increased pathogen load of the putative gill pathogen *Ca.* B. cysticola after thermal delousing. The overall percentage of gill tissue affected was relatively low, generally estimated to be <2%, and thus the clinical significance of these lesions remains to be established.

Most vascular and hyperplastic lesions were found at 1 and 2 days and 8 and 9 days after mechanical and thermal delousing, respectively. The distribution and character of lesions also differed between the two
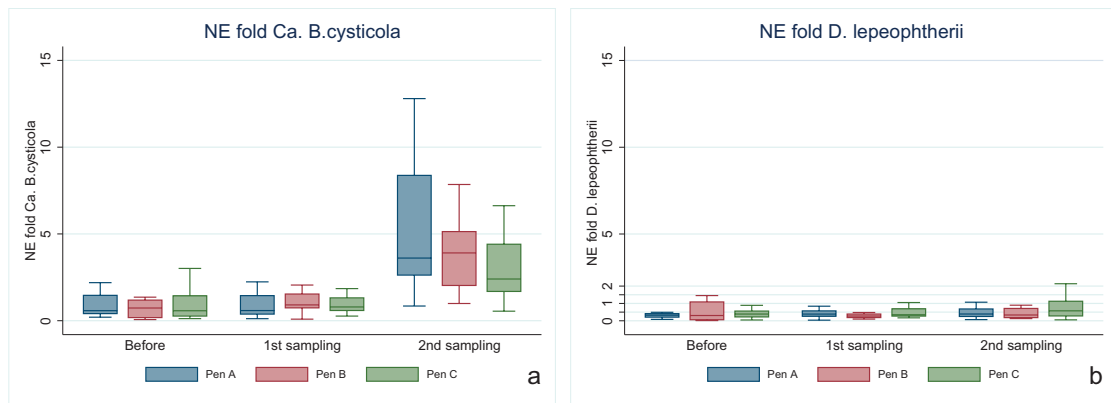
Fig. 5. Normalized (NE) fold change for gill pathogens *Ca.* B. cysticola and *D. lepeophtherii.* Box and whisker plots show lesions per pen and timepoint ($n = 18$–30) for a) *Ca.* B. cysticola and b) *D. lepeophtherii.* Outliers are excluded for clarity.

Table 7

Thermal delousing. Results of statistical analysis for fold change of genes and pathogens detected by PCR-analysis. The linear regression model for each includes sampling point ($n = 44$–54) and fish group/pen (n = 44–54, total $n =$ 142). NS – not significant.

Gene/ agent	Time	Coefficient	P > |t|	95% Conf. Interval
Ca. B. cysticola	Before			
	1st sampling	0.36	0.029	(0.04, 0.69)
	2nd sampling	1.67	0.000	(1.38, 1.96)
Heat shock protein 70	Before			
	1st sampling	0.32	0.000	(0.21, 0.42)
	2nd sampling	0.18	0.002	(0.06, 0.29)
Tumor necrosis factor-α	Before			
	1st sampling	1.11	0.000	(0.91, 1.32)
	2nd sampling	0.85	0.000	(0.63, 1.06)
Interleukin-1β	Before			
	1st sampling	0.58	0.000	(0.45, 0.71)
	2nd sampling	0.24	0.001	(0.10, 0.38)
Interleukin-10	Before			
	1st sampling	0.10	0.019	(0.02, 0.18)
	2nd sampling	−0.49	0.000	(-0.57, -0.40)
Interleukin-4/13α	Before			
	1st sampling	0.86	0.000	(0.69, 1.03)
	2nd sampling	0.57	0.000	(0.39, 0.74)
Interleukin-4/13β2	Before			
	1st sampling	−0.25	0.005	(-0.43, -0.08)
	2nd sampling	−0.44	0.000	(-0.62, -0.25)
Hypoxia-inducible factor-1α	Before			
	1st sampling	−0.12	0.175	(-0.29, -0.05)
	2nd sampling	−0.34	0.000	(-0.53, -0.16)

Table 8

Thermal delousing. Results of statistical analysis for fold change of genes examined by PCR-analysis. Kruskal-Wallis tests were performed to assess whether there was a significant difference between time point for each pen separately. 13–18 fish were included per pen/time point. Total $n = 142$. NS – not significant.

Pen	Time compared	Interferon-γ *p*-value	Transforming growth factor-β *p*-value	Epidermal growth factor receptor *p*-value
	0–1 days	0.000	0.000	0.000
A	0–8 days	0.013	0.102	0.294
	1–8 days	0.000	0.000	0.000
	0–1 days	0.004	0.000	NS
B	0–8 days	0.008	0.237	NS
	1–8 days	0.000	0.000	NS
	0–2 days	0.003	0.000	0.002
C	0–9 days	0.011	0.445	0.047
	2–9 days	0.000	0.000	0.130

The observed vascular and hyperplastic lesions will have a negative effect on the overall gill function. Thrombosis and aneurysms of lamellar sinusoids and hyperplasia of lamellar epithelium reduce the surface available for oxygen exchange, hampers excretory and osmoregulatory functions and leads to initiation of reactive and reparative responses in the gill tissue (Speare et al., 1999; Wolf et al., 2015). The time necessary for resolution of gill injuries likely depends on the extent and type of injury, environmental factors, potential pathogens present, fish state and management operations occurring after the initial insult (Speare and Ferguson, 2006; Speare et al., 1999). While hyperplastic lesions can resolve by shedding and apoptosis of excess epithelial cells, formation of thrombi and aneurysms can be associated with necrosis and loss of pillar cells so that cell migration and regeneration of pillar cells is necessary to restore a functional vascular lumen (Speare and Ferguson, 2006; Speare et al., 1999). Resolution of hyperplastic lesions have been observed from 7 days to 5 weeks after removal of the inciting microorganism (Adams and Nowak, 2001; Castrillo et al., 2021; Harris et al., 2004; Kudo and Kimura, 1983), but Speare et al., (1999) showed that lamella severely damaged by hydrogen peroxide had not been completely regenerated and functional vascular sinusoids were lacking 3 weeks after exposure. Further, the observation of masses of fibrovascular tissue or even cartilage partly or completely replacing normal lamellar sinusoids may suggest that restoration to normal shape and function could be incomplete or delayed in some cases (Østevik et al., 2021).

Norwegian farmed Atlantic salmon may undergo multiple treatments

treatment systems. After mechanical delousing all categories of vascular lesions and hyperplastic lesions were most often located in filament tips, there was an overlap of hyperplasia and vascular damage, and aneurysms were more frequently observed than thrombi in normal diameter sinusoids. Infiltration of presumed leukocytes in the hyperplastic lesions were mostly limited or absent and lamellar epithelial hyperplasia in these fish could represent a reactive response to the vascular damage. Randomly distributed thrombi in normal diameter sinusoids without associated hyperplastic lesions were the dominating type of vascular lesion observed after thermal delousing. These observations indicates that gill lesions developed during and rapidly after mechanical delousing, and mechanical trauma is a likely cause of the injury. In contrast, the gradual increase in extent and the tissue distribution of thrombi after thermal delousing suggests that mechanisms other than mechanical trauma are involved though further work is necessary to determine this.
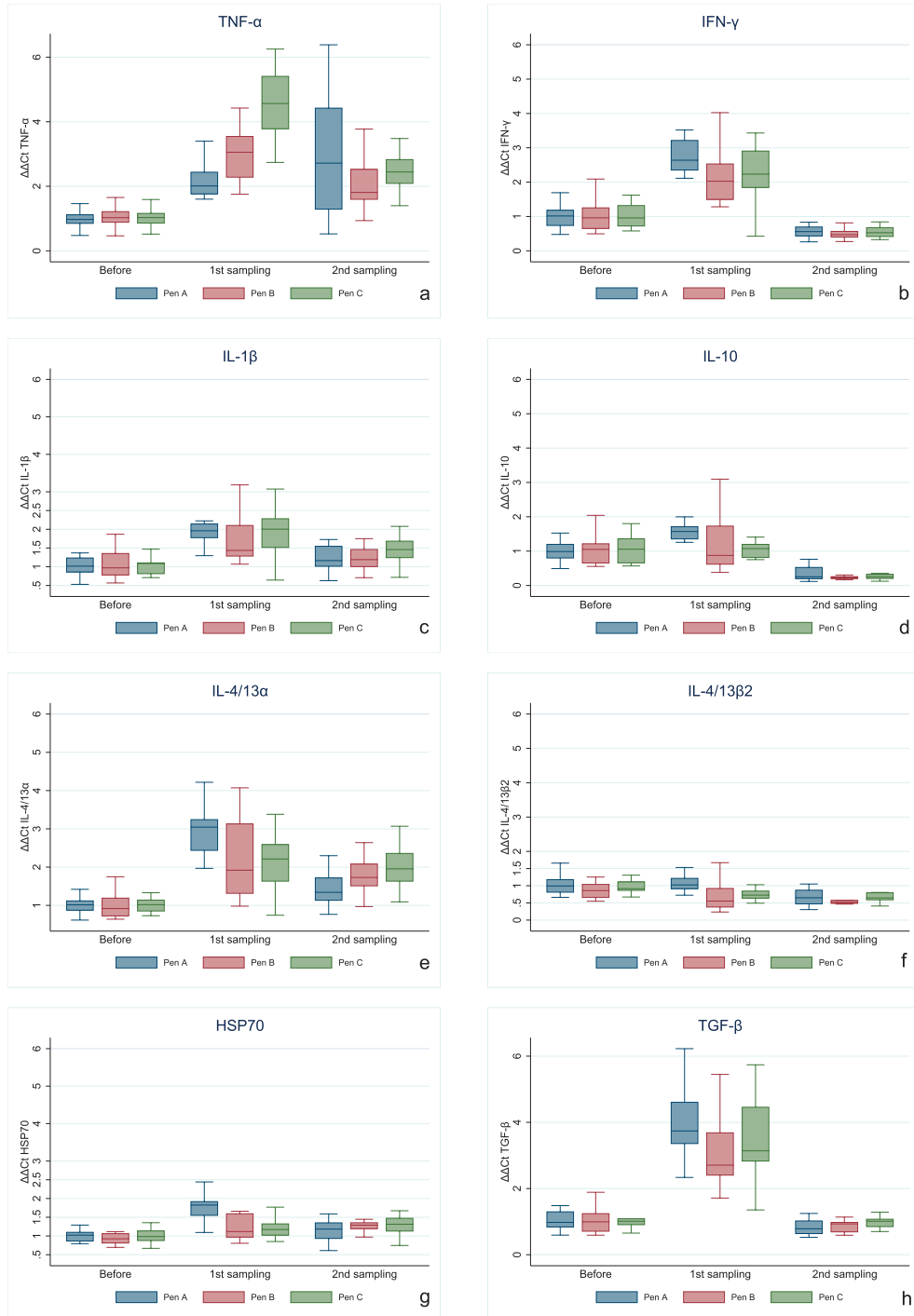
Fig. 6. $2^{-\Delta\Delta Ct\ values}$ for genes examined in the study. Box and whisker plots shown per pen and timepoint ($n = 13$–18) for a) TNF-α, b) IFN-γ, c) IL-1β, d) IL-10, e) IL-4/13α, f) IL-4/13β2, g) HSP70, h) TGF- β, i) EGFR and j) HIF-α. Outliers are excluded for clarity.
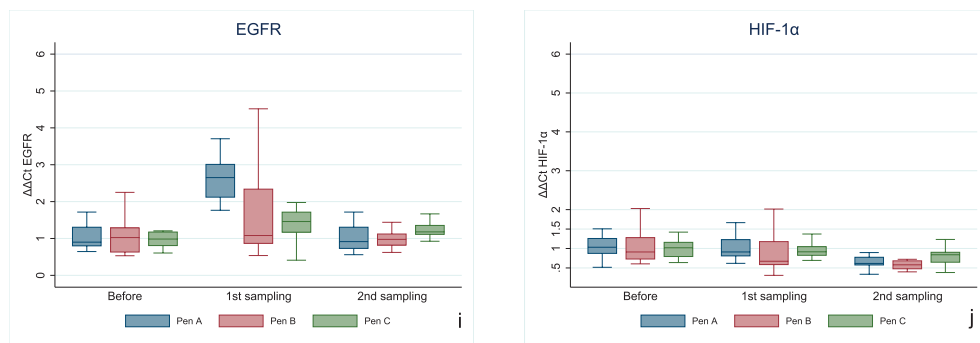
Fig. 6. (*continued*).

for sea lice infestation during a production cycle. The health status at onset of treatment can vary and therefore impact of the treatment on fish health may vary (Sommerset et al., 2021). In the data reported by Sviland Walde et al., (2021) the mean number of sea lice treatments was 3 per fish group and production cycle, and it took 28 and 31 days (median) from mechanical and thermal delousing, respectively, until any new treatment for sea lice was performed (C. Sviland Walde, personal communication, October 7, 2021). In a recent longitudinal study, the number of delousing operations (excluding in-feed treatment) for one production cycle ranged from 0 to 11 between fish groups, with a median of 6 treatments per group (Østevik et al., 2022). Repeated treatments may have a cumulative effect on gill lesions over time.

A number of laboratory studies and field trials reported no clear increase in the severity of gill injuries after heated water exposure and thermal or mechanical delousing (Bentzen et al., 2018; Ellingsen and Moljord, 2019; Erikson et al., 2018; Grøntvedt and Kristensen, 2018; Grøntvedt et al., 2015; Kvåle, 2020; Mangor-Jensen et al., 2017; Moltumyr et al., 2021; Roth, 2016; Salvesen et al., 2021). In contrast to these observations, we found an increased percentage of vascular and hyperplastic lesions after both thermal and mechanical delousing, although a small percent of the gill tissue was affected. Our findings are supported by (Gismervik et al., 2017) who reported a significant increase in grossly visible gill hemorrhages after FLS treatment. However, a later study of an improved FLS system found only mild gill hemorrhage and concluded that the new system significantly reduced the risk of gill hemorrhage (Grøntvedt and Kristensen, 2018). Two studies reported gill aneurysms (described by Gismervik et al., 2019 as bleeding), hemorrhage and necrosis after warm water exposure in the laboratory or thermal delousing in the field (Gismervik et al., 2019; Jørgensen and Rød, 2019), but increased severity of gill aneurysms and hemorrhage in the field trial was only found in fish with shortened opercula suggesting mechanical injuries could be the reason. An increase in the severity of gill thrombosis and hyperplasia after thermal delousing has not been reported previously.

The discrepancies between the current study and previous studies could be explained by differences between fish groups, environmental factors, delousing methods and equipment used, but could also be related to the different methods used to assess gill health (Adams et al., 2004; Caswell et al., 2018; Gibson-Corley et al., 2013). Quantitative, or semi-quantitative microscopic assessment of gill tissue was not performed in any of the previous studies examining potential health and welfare effects of mechanical delousing nor in the majority of field trials examining thermal delousing (Bentzen et al., 2018; Gismervik et al., 2017; Grøntvedt and Kristensen, 2018; Grøntvedt et al., 2015; Nilsen et al., 2010; Roth, 2016; Salvesen et al., 2021). In addition, gross examination and scoring of skin, eye, gill and fin lesions, and percent mortality after treatment was the main parameters used to assess effect of delousing in previous studies. Thus, direct comparison between

studies is difficult. The histopathological assessment performed in the current study gives a high degree of detail and as such smaller increases in extent of tissue damage can be detected compared to an ordinal four- or five-tier scoring system or when performing gross assessment of the gill tissue (Adams et al., 2004; Birkebak and Mann, 2019; Meyerholz and Beck, 2018). This fact should also be considered as a possible explanation for the discrepancies observed.

The increased expression HSP70, TNF-α, TGF-β, IL-1β, IL-4/13α, IL-10 and IFN-γ at 1 and 2 days after treatment indicate that thermal delousing induces responses related to cell stress, regulation and promotion of inflammation, and repair. However, at 8 and 9 days after delousing downregulation or return to pre-treatment levels of anti-inflammatory cytokines IL-10 and TGF-β while pro-inflammatory cytokines TNF-α, IL-1β and IL-4/13α remain upregulated. This suggests that pro-inflammatory responses are maintained over the observation period. IL-1β, TNF-α and IFN-γ are pleiotropic cytokines with a multitude of functions and are elicited through tissue-damaging events, infectious or mechanical insults or a combination of these (Idriss and Naismith, 2000; Kaneko et al., 2019; Schroder et al., 2004). Both TNF-α and IL-1β lead to endothelial activation and can have prothrombotic effects (Pircher et al., 2012; Yoshida et al., 2010), thus a sustained inflammatory response could be a factor in the increase of vascular lesions observed after thermal delousing.

Further, IL-4/13α and IL-4/13β2 are important mediators in allergic and inflammatory diseases and can also skew immune responses towards a Th2-profile (Bottiglione et al., 2020; Junttila, 2018; Wang et al., 2016). IL-13 can induce proliferation of human bronchial epithelial cells by releasing TGF-β that subsequently binds and activates EGFR (Booth et al., 2007). EGFR exerts critical functions in epithelial cell physiology, such as cell proliferation, survival, and differentiation (Dong et al., 1999; Sigismund et al., 2018). Additionally, TNF-α, mechanical damage, toxins and irritants can induce EGFR-expression in mammalian airway epithelial cells (Booth et al., 2007; Burgel and Nadel, 2004). In Atlantic salmon with amoebic gill disease, IL-4/13α, IL-4/13β2, IL-1β, TNF-α and EGFR have been shown to be upregulated in hyperplastic gill lesions characteristic of the disease (Benedicenti et al., 2015; Bridle et al., 2006; Marcos-Lopez et al., 2018; Morrison et al., 2012; Morrison et al., 2007; Pennacchi et al., 2014). However, to what extent the hyperplastic responses in the current study are linked to upregulation of inflammatory cytokines or EGFR remains to be determined.

An increase in HSP70 expression and protein levels can occur in a response to heat stress (Ackerman et al., 2000; Basu et al., 2001; Buckley et al., 2006; Fangue et al., 2006), but can also be induced by a range of other insults including bacteria, virus, parasites and toxins (Ackerman and Iwama, 2001; Marcos-López et al., 2017; Olsvik et al., 2014; Zhang et al., 2013). The upregulation of HSP70 in the current study can represent a protective response towards an adverse stimulus (Jacquier-Sarlin et al., 1994), though the role of HSP70 with regards to the

observed gill lesions, if any, is unclear. Further, the exposure to heat can by itself impose stress with resulting upregulation of HSP70 but it is difficult to discriminate between effects of heat per se and the stress imposed by the handling in this study.

Hypoxia inducible factor-1alpha is a transcription factor and a master switch for hypoxia-induced responses (Nikinmaa and Rees, 2005), including responses of tissue repair (Cadiz and Jonz, 2020). The lack of differential expression or only slight downregulation of HIF-1α after delousing suggests that the delousing operation and the observed gill lesions were not associated with significant tissue hypoxia nor induced HIF-1α regulated reparative responses in the gill tissue.

An examination of the effect of commercial thermal delousing on gill gene expression has not been performed previously to the best of our knowledge, and comparison with other studies is therefore not possible. The low correlation between the extent of gill lesions and fold change of gene expression was somewhat surprising as we expected to find a link between at least some genes and the degree of gill histopathology. An explanation could be that selected genes are not important for the development or progression of the lesions observed. In hindsight, genes involved in hemostasis and endothelial activation like platelet derived growth factor, thromboxane A₂, tissue factor or inducible nitric oxide synthase might have yielded other results (Kumar et al., 2015). Lack of correlation could also be a timing issue as differential regulation of genes can occur prior to or after development of observable gill lesions (Sollid et al., 2006). Lastly, it has been shown that correlation between mRNA levels and the abundance of the proteins they encode are variable, and post-transcriptional regulation, translational regulation and protein degradation could affect the actual amount of active protein present in the tissue (Vogel and Marcotte, 2012). As such a mismatch between mRNA and active protein levels in our gill samples is also possible, though this was not explored in the current study.

The high prevalence of *Ca.* B. cysticola and *D. lepeophtherii*, epitheliocysts, and gill lesions possibly associated with of *D. lepeophtherii* and/or *Ca.* B. cysticola infection was not unexpected. Earlier studies have shown *Ca.* B. cysticola and *D. lepeophtherii* infection to be ubiquitous in Atlantic salmon at sea in Southern Norway and Ireland (Downes et al., 2018; Kvåle, 2020; Nylund et al., 2011; Steinum et al., 2010). Both *Ca.* B. cysticola and *D. lepeophtherii* have been associated with subepithelial inflammation and necrosis (Gjessing et al., 2021; Weli et al., 2017), while *D. lepeophtherii* has also been associated with ballooning degenerative cells containing pigmented material (Gjessing et al., 2019; Gjessing et al., 2021). The pathogenicity of these agents remains unclear and both agents have been identified in fish with and without gill disease (Mitchell et al., 2013; Nylund et al., 2011; Rodger et al., 2011; Steinum et al., 2010; Toenshoff et al., 2012). There is no clear evidence for a role of *Ca.* B. cysticola and *D. lepeophtherii* in development of gill thrombi or aneurysms (Gjessing et al., 2019; Gjessing et al., 2021; Gunnarsson et al., 2017; Matthews et al., 2013; Nylund et al., 2010; Steinum et al., 2015; Weli et al., 2017), but it cannot be ruled out that these agents contributed to the increased severity of hyperplastic lesions observed after delousing in the current study, although low correlation between pathogen load detected by RT-qPCR and hyperplastic lesions found in the fish undergoing thermal delousing does not support this. The increased load of *Ca.* B. cysticola and increased prevalence of gill pathogens and tissue lesions possibly associated with gill pathogens *Ca.* B. cysticola and *D. lepeophtherii* after delousing could represent an effect of the treatment, as stress and increased cortisol levels can lead to increased susceptibility to infection and reduced ability of the fish immune system to limit proliferation of infectious agents (Pickering and Pottinger, 1985). In support of this notion is a recent study showing that Atlantic salmon with "hidden" *Yersinia ruckeri* infections can excrete significant amounts of bacteria during thermal delousing (Strand et al., 2021). However, an increase in pathogen load of *Ca.* B. cysticola or *D. lepeophtherii* was not consistently observed after delousing in a recent field trial, nor was there an increased load of other infectious agents after treatment (Kvåle, 2020). Further, in the current study down-

regulation of pro-inflammatory cytokines was not evident after delousing, except for IFN-γ and IL-4/13β2.

It is unclear if the gill lesions contributed to the increased mortality rates observed. Fish dying during or after treatment were not sampled due to the rapid autolysis of gill tissue *postmortem* (George et al., 2016; Munday and Jaisankar, 1998). Because of this we do not know if fish that died had other injuries or diseases, or more severe gill pathology than the fish that survived and were sampled for the study. The fact that the degree of gill pathology was not substantially different in the fish with the highest mortality after delousing could suggest that the gill pathology was not an important factor for the observed treatment related mortality. The delta mortality rates reported here were below the average overall mortality rates reported for thermal and mechanical delousing earlier (Sviland Walde et al., 2021) except for one pen (mechanical delousing pen D).

The low number of pens included, and the limited follow up time post-delousing are important limitations of this study and previous studies examining health and welfare impacts of mechanical and thermal delousing. Further, the fish in both of our trials had been at sea for 10 months and treatment and sampling were performed in summer and autumn which are the period when gill diseases are more commonly observed. Thus, environmental factors and pathogens present in the population could have affected the outcome of the study. It is not possible to determine to what extent the lesions and differential gene expression in the gill tissue are caused by the increased water temperature or flushing per se relative to the impact of crowding and pumping of the fish. However, criteria for site selection were set to ensure that presumably healthy fish and sites without problems related to jellyfish or phytoplankton or injuries from earlier delousing events were included. We performed sampling over a limited time span and included fish from a single site for each treatment to have the best chance of detecting lesions related to delousing and reducing the likelihood of other insults that could lead to development of lesions. We aimed to detect effects of delousing as it is performed in a commercial production environment, and design of the study does not allow us to separate the potential effects of crowding and handling from the effect of the specific delousing methods used. However, sampling fish during real-life treatment events will give the best picture of the total health impact of thermal and mechanical delousing operations. More studies are necessary to further explore how thermal and mechanical delousing impacts fish health and to what extent the observed gill lesions will resolve or can accumulate with repeated delousing operations. Knowledge about the health effects of non-medicinal delousing and potential differences between the delousing methods will be important for the choice of salmon lice management in the future.

Authorship statement

All persons who meet authorship criteria are listed as authors, and all authors certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript. The name of each author must appear at least once in each of the three categories below. Category 1 Conception and design of study: LØ, MS, MA; acquisition of data: AS, LØ, MA, CX, FM, ØE; analysis and/or interpretation of data: LØ, MS, AN, ØE.

Category 2 Drafting the manuscript: LØ; revising the manuscript critically for important intellectual content: MA, KIL, MS, AN, ØE, HR, FM, CX, AS.

Category 3 Approval of the version of the manuscript to be published (the names of all authors must be listed): MA, KIL, MS, AN, HR, AS,CX, LØ, ØE, FM.

Declaration of Competing Interest

LØ, KIL and MA are employed by Pharmaq Analytiq AS, a business

ultimately owned by Zoetis Inc. Zoetis Inc. is also the ultimate owner of Pharmaq AS, a vendor of compounds for medicinal sea lice treatment. AS is employed by Måsøval AS that owns and regularly use FLS delousing units for control of sea lice levels, while FM is employed by Mowi ASA that owns and regularly use Thermolicer and flusher units for delousing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2022.738019.

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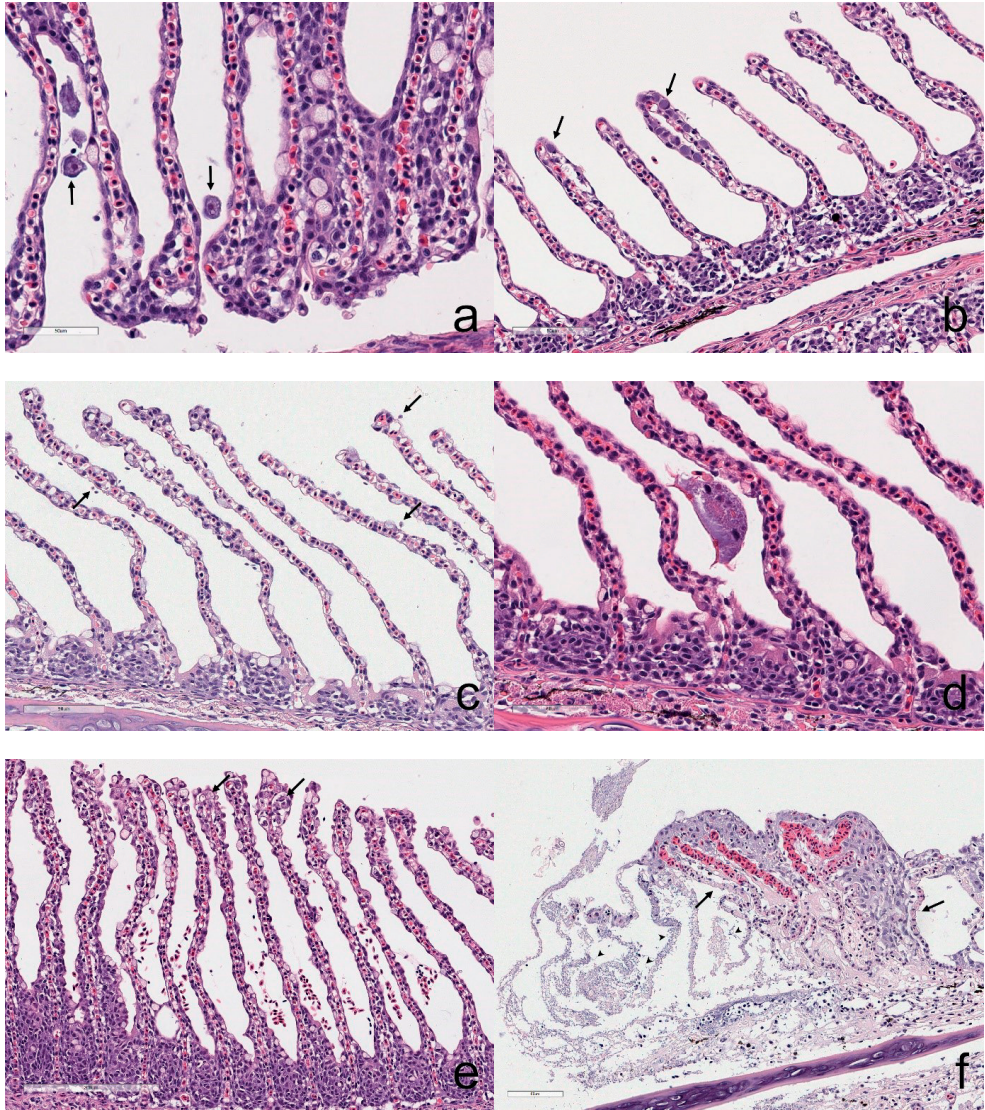
Figure S1. Pathogens or pathogen-associated lesions. All tissues stained with haematoxylin and eosin. a) Amoeba (arrows) and mild lamellar epithelial hyperplasia. b) Intracellular bacteria "epitheliocysts" (arrows), c) *Ichthyobodo* spp. "costia" (arrows). d) *Trichodina* spp. e) Subepithelial inflammation, necrosis and pale, granulated pigmented material (arrows). f) Filamentous bacteria (arrowheads) and necrosis with loss of normal tissue structure, also lamellar epithelial hyperplasia, haemorrhage, and thrombi (arrows).

Supplementary file 1.

Pen	Before	Sampling 1	Sampling 2
A	Histology n=30 *D. lep* & *Ca.* B. cys n=18 *N. per* & SGPV n=5 Gene expression n=18	Histology n=29 *D. lep* & *Ca.* B. cys n=18 *N. per* & SGPV n=5 Gene expression n=18	Histology n=30 *D. lep* & *Ca.* B. cys n=30 *N. per* & SGPV n=30 Gene expression n=18
B	Histology n=30 *D. lep* & *Ca.* B. cys n=18 *N. per* & SGPV n=5 Gene expression n=13	Histology n=30 *D. lep* & *Ca.* B. cys n=18 *N. per* & SGPV n=5 Gene expression n=18	Histology n=30 *D. lep* & *Ca.* cys n=30 *N. per* & SGPV n=25 Gene expression n=13
C	Histology n=30 *D. lep* & *Ca.* B. cys n=18 *N. per* & SGPV n=5 Gene expression n=13	Histology n=30 *D. lep* & *Ca.* B. cys n=18 *N. per* & SGPV n=5 Gene expression n=18	Histology n=30 *D. lep* & *Ca.* B. cys n=30 *N. per* & SGPV n=25 Gene expression n=13

Table S1. Overview of samples and analysis performed for the thermal delousing study.

Gene/Pathogen	Target	Sequence	Reference
Elongation factor-1α	Salmonid ELF	Fwd. GGCCAGATCTCCCAGGGCTAT Rev. TGAACTTGCAGGCGATGTGA Probe HEX-CCTGTGCTGGATTGCCATACTG-BHQ1	(Bruno, et al., 2007)
Neoparamoeba perurans	18S rRNA	Fwd. GTTCTTTCGGGGAGCTGGGAG Rev. GAACTATCGCCGGCACAAAAG Probe FAM-CAATGCCATTCTTTTCGGA-MGB	(Fringuelli, et al., 2012)
Ca. Branchiomonas cysticola	16s RNA	Fwd. AATACATCGGAACGTGTCTAGTG Rev. CCATCAGCCGCTCATGT Probe FAM-CTCGGTCCCAGGCTTTCCTCTC-BHQ1	(Mitchell, et al., 2013)
Paranucleospora theridion / Desmozoon lepeophtherii	16s rRNA	Fwd. CGGACAGGGAGCATGGTATAG Rev. GGTCCAGGTTGGGTCTTGAG Probe FAM-TTGGCGAAGAATGAAA-MGB	(Nylund, et al., 2010)
Salmon Gill Pox Virus	major capsid protein	Fwd. ATCCAAAATACGGAACATAAGCAAT Rev. CAACGACAAGGAGATCAACGC Probe FAM-CTCAGAAACTTCAAAGGA-MGB	(Gjessing, et al., 2015)

Table S2. Primer, probe, target, and references for assays used for pathogen qPCR-analysis.

Assay	Slope	Efficiency
Neoparamoeba perurans	-3,289	101 %
Ca. Branchiomonas cysticola	-3,366	98 %
Desmozoon lepeophtherii	-3,967	79 %
Salmon gill poxvirus (SGPV)	-3,5	93 %
N.perurans Ef1α	-3,474	94 %
Ca. B. cysticola Ef1α	-3,392	97 %
D.lepeophtherii Efα	-3,352	99 %
SGPV Ef1α	-3,135	108 %

Table S3. Efficiency and slope of pathogen qPCR-assays.

Assay	Slope	Efficiency
β-actin	-2,76	130.31%
Heat shock protein 70	-2,852	124.20%
Hypoxia-inducible factor 1-α	-3,185	106.05%
Interleukin 1-β	-3,098	110.28%
Tumor necrosis factor- α (TNF- α)	-2,853	124.13%
Interferon-γ (INF- γ)	-3,177	106.43%
Interleukin-10	-3,197	105.49%
Transforming growth factor β (TGF-β)	-3,104	109.97%
Interleukin 4/13α (il4/13α)	-3,147	107.86%
Interleukin 4/13β2 (il4/13β2)	-3,175	106.52%
Epidermal growth factor receptor (EGFR)	-3,242	103.45%

Table S4 Efficiency and slope of qPCR-assays. The efficiency of each assay was calculated by analysing a 10-fold dilution series in duplicates.

Site	Pen	ΔMrate 1 day	ΔMrate 7 days	ΔMrate 14 days	ΔMrate 2nd week
Thermal delousing	A	0.000318758	0.000156651	8.81853E-05	9.91079E-06
	B	0.001134383	0.000587201	0.000342519	6.28063E-05
	C	0.001820637	0.001172057	0.000649782	5.28155E-05
Mechanical delousing	D	0.002156983	0.000875792	0.000494509	5.87596E-05
	E	-3.62168E-05	0.000352765	0.000357511	0.000363115
	F	0.000835282	0.000981454	0.000624397	0.000216339

Table S5. ΔMrate at selected time points after delousing. A positive value indicates an increased mortality rate after delousing compared to the mortality rate in the 7 days before delousing. A negative value is consistent with a decreased mortality rate after delousing.

Site	Lesion	Before Counts Mean Median Min-Max	Before % Mean Median Min-Max	1st sampling Counts Mean Median Min-Max	1st sampling % Mean Median Min-Max	2nd sampling Counts Mean Median Min-Max	2nd sampling % Mean Median Min-Max
Thermal delousing	Acute vascular lesions	28.84 19 1-137	0.21 0.16 0.01-0.83	28 18 2-182	0.23 0.15 0.02-1.63	27.59 13.5 1-350	0.19 0.09 0.01-2.19
	Thrombi	27.14 23 2-109	0.21 0.17 0.01-0.87	36.11 28 0-128	0.27 0.22 0-0.75	63.66 55 1-237	0.44 0.41 0.01-1.48
	Reactive and repairing vascular lesions	20.63 17 1-93	0.15 0.13 0.01-0.60	20.84 14 0-212	0.16 0.12 0-1.68	46.41 30 1-637	0.30 0.09 0.01-3.82
	Sum vascular lesions	76.62 68.5 14-249	0.58 0.51 0.10-1.76	84.96 71 14-305	0.68 0.63 0.11-2.42	137.66 122 8-712	0.94 0.85 0.08-4.27
	Hyperplasia	10.77 4 0-106	0.08 0.03 0-0.75	25.17 5 0-700	0.19 0.04 0-4.83	44.17 12 0-652	0.30 0.09 0-3.89
Mechanical delousing	Acute vascular lesions	25.53 17.5 0-277	0.21 0.14 0-2.55	46.41 25 0-558	0.55 0.30 0-6.20	27.76 22 3-214	0.22 0.15 0-2.52
	Thrombi	14.53 12 0-67	0.11 0.10 0-0.49	26.71 19.5 0-139	0.31 0.20 0-1.66	30.1 23.5 1-121	0.22 0.18 0.01-0.88
	Reactive and repairing vascular lesions	12.2 8 0-55	0.1 0.07 0-0.56	41.66 16.5 0-532	0.50 0.19 0-5.70	53.88 25 1-278	0.39 0.21 0.01-2.15
	Sum vascular lesions	63.18 52.5 4-341	0.42 0.33 0.06-2.90	114.78 66.5 2-1249	1.36 0.68 0.03-13.39	111.73 71 8-379	0.82 0.53 0.05-3.2
	Hyperplasia	10.98 6 0-72	0.09 0.5 0-0.65	20 2 0-312	0.25 0.03 0-4.43	34.96 13 0-348	0.26 0.1 0-2.78

Table S6. Summary of histology lesions recorded as counts and percent for all pens across the three sampling points. A total of 90 fish (n = 30 per pen) was sampled per time point except for one pen at sampling 2 after thermal delousing when 29 fish was sampled.

Site	Lesion/organism	Before *# and % fish* *n = 90*	1st *sampling* *# and % fish* *n =89 - 90*	2nd *sampling* *# and % fish* *n = 90*
Thermal delousing	Epithelial cell necrosis	51 (57%)	43 (48%)	56 (62%)
	Lamellar adhesion	6 (7%)	10 (11%)	29 (32%)
	Lamellar oedema	18 (20%)	4 (5%)	19 (21%)
	Inflammation filament	52 (58%)	46 (52%)	45 (50%)
	Subepithelial inflammation	18 (20%)	28 (31%)	56 (62%)
	Epitheliocysts	72 (80%)	80 (90%)	87 (97%)
	Amoeba	1 (1%)	1 (1%)	0 (0%)
	Bacteria	0 (0%)	0 (0%)	0 (0%)
	Ichthyobodo sp.	1 (1%)	0 (0%)	1 (1%)
	Trichodina sp.	37 (41%)	49 (54%)	56 (62%)
Mechanical delousing	Epithelial cell necrosis	24 (27%)	11 (12%)	25 (28%)
	Lamellar adhesion	16 (18%)	4 (4%)	11 (12%)
	Lamellar oedema	2 (2%)	6 (7%)	6 (7%)
	Inflammation filament	65 (72%)	38 (42%)	69 (77%)
	Subepithelial inflammation	13 (14%)	17 (19%)	25 (28%)
	Epitheliocysts	74 (82%)	81 (90%)	75 (83%)
	Amoeba	0 (0%)	0 (0%)	0 (0%)
	Bacteria	1 (1%)	0 (0%)	3 (3%)
	Ichthyobodo sp.	2 (2%)	6 (7%)	10 (11%)
	Trichodina sp.	0 (0%)	1 (1%)	0 (0%)

Table S7. Summary of histology lesions and pathogens recorded as dichotomous variables for all pens across the three sampling points. A total of 270 fish were sampled during the mechanical delousing, while 269 fish were sampled during the thermal delousing.

Gene	Before Mean Median Min-Max	1st sampling Mean Median Min-Max	Sampling 2 Mean Median Min-Max
Heat shock protein 70	1.03 1.01 0.67-2.28	1.43 1.29 0.80-2.75	1.22 1.28 0.61-1.67
Tumor necrosis factor-α	1.07 1.00 0.47-2.69	3.41 3.06 1.60-11.68	3.20 2.29 0.52-27.27
Interleukin 1β	1.05 1.02 0.53-1.94	1.87 1.94 0.64-3.18	1.36 1.26 0.63-3.28
Interleukin 10	1.07 1.02 0.49-2.04	1.30 1.22 0.21-3.22	0.30 0.25 0.12-1.15
Interleukin 4/13α	1.04 1.01 0.62-2.50	2.59 2.42 0.74-6.02	2.00 1.63 0.77-7.22
Interleukin 4/13β2	1.09 0.93 0.55-3.23	0.86 0.79 0.15-2.54	0.74 0.60 0.31-2.97
Hypoxia-inducible factor 1-α	1.05 1.02 0.52-2.11	0.98 0.89 0.17-2.45	0.83 0.64 0.34-4.07
Interferon-γ	1.11 1.00 0.48-2.82	2.43 2.36 0.43-6.52	0.60 0.52 0.26-2.08
Transforming growth factor-β	1.05 0.99 0.59-2.35	3.56 3.41 1.35-6.98	0.91 0.93 0.49-2.03
Epidermal growth factor receptor	1.07 0.97 0.53-2.72	1.95 1.77 0.41-5.31	1.40 1.08 0.56-8.93

Table S8. $2^{-\Delta\Delta Ct\ values}$. 13-18 fish included per pen/time point. n = 142.

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Supplementary file S2: Histopathology protocol

This two-step assessment system is based on first estimating the number of lamella available for evaluation in each sample, and then using this estimate to calculate the estimated percent of gill tissue affected by different lesions.

Step 1. Estimate the number of secondary lamellae in the sample.

In a small subset of fish representative of the samples to be analyzed one filament was measured and the number of lamellae was counted (thermal delousing sample set n=9 gills, n=3 per pen; mechanical delousing sample set n=12 gills, n=4 per pen). This filament should have adequate orientation (i.e., lamellae present on at least one side of the filament along the entire length of the filament). Then the number of lamellae per millimeter filament was calculated for each sample (#lamellae/filament length in millimeter) and the mean number lamella per mm filament across all samples for each sample set was used in further calculations to estimate the number of lamella available for examination in each gill sample.

The mean number of lamellae per millimeter filament for thermal delousing samples was 33,17 with a range of 31,23-35,62, while it was 32,87 with a range of 31-34,50 for mechanical delousing samples.

For each sample the number of filaments was counted, and a filament deemed representative of the mean filament length in the sample was measured. All filaments were then assessed for quality of plane of section and the number of filaments with good, average, and poor plane of section was counted. For a filament with good plane of section lamellae were present on both sides along the majority (> 80%) of the filament. In filaments with average plane of section

lamellae were present on both sides along 50 to 80% of the filaments, while lamellae were present on both sides on less than 50% of the filament as poor plane of section. Based on the above information, the estimated number of lamellae available for assessment in each sample was calculated as follows:

1. $\#Lamella\ per\ filament\ =$ length of average filament (mm) $\times$ $mean\ \#lamella\ per\ mm\ filament$

2. $\#Lamella\ in\ sample = (\#filament\ in\ good\ plane\ of\ section\ \times$ $\#lamella\ per\ filament) +\ (\#filaments\ in\ average\ plane\ of\ section\ *$ $\#lamellae\ per\ filament\ * 0.8) + (\#filaments\ in\ poor\ plane\ of\ section\ *$ $\#lamellae\ per\ filament\ * 0.5)$

Step 2. Estimating the % gill tissue affected by a given gill lesion.

For each of the categories of gill lesions that were recorded quantitatively and semi-quantitatively the number of lamellae affected by each lesion were counted. The percent of affected lamellae was subsequently calculated as:

$$Percent\ affected\ lamellae\ = \frac{number\ of\ lamellae\ with\ a\ lesion}{estimated\ number\ of\ lamellae\ in\ the\ sample} \times 100$$

Counts and percent of gill hyperplasia, acute vascular lesions, thrombi, reactive and reparative vascular lesions, and necrosis was recorded. These lesions were defined as follows:

Acute aneurysms

- Dilation of lamellar vascular sinusoids with rupture and loss of pilar cells, but without proliferation of lamellar epithelium, fibrin deposition or thrombosis of the affected vessel.

Haemorrhage

- The presences of extravascular erythrocytes within the gill tissue. Red blood cells free between lamella or filaments are not counted as hemorrhage.

Thrombi

- Aneurysms, with fibrin deposition, but without proliferation of lamellar epithelium or infiltration of intact cells into the thrombosed vessel.
- Thrombi in lamellar sinusoids with normal diameter, but without proliferation of lamellar epithelium or infiltration of intact cells into the thrombosed vessel.

Reactive and reparative vascular lesions

- Aneurysms or thrombi with 1 or more of:
  - Proliferation of surrounding lamellar epithelium (at least 2 cell-layers thick)
  - Recanalization of thrombus
  - Infiltration of intact cells (presumed fibroblasts or endothelial cells) into the thrombosed vessel
  - Thickening of the vascular basal lamina
  - Infiltration of fibrovascular tissue with or without obliteration of the vessel lumen

Hyperplasia

- Proliferation of lamellar epithelium cells to the extent that at least 80% of the interlamellar space is filled with hyperplastic cells. Lamella on each side of the affected interlamellar spaces are counted.
- If a combination of hyperplasia and leukocytic infiltrates are present the lesion is also counted as hyperplasia.

Necrosis

- Cell death of a least one secondary lamella, including epithelium and pilar cells.

In addition, presence, or absence of the following lesions (or pathogens) were recorded as 0 or 1:

- Epithelial cell necrosis or apoptosis
  - Lamellar epithelial cells with hypereosinophilic cytoplasm and/or nuclear pyknosis, karyorrhexis or karyolysis
  - Lamellar epithelial cells with margination or clumping of nuclear chromatin
- Adhesion of lamellae
  - Adhesions between one or more lamellae
- Hypertrophy/swelling of epithelial cells
  - Cuboidal or swollen gill epithelium lacking signs of necrosis or apoptosis – i.e. not hypereosinophilic cytoplasm or nuclear pyknosis, karyorrhexis or karyolysis
- Lamellar oedema/"lifting"
  - Accumulation of eosinophilic homogenous or granular material (presumed fluid) between the lamellar basal lamina and lamellar epithelial cells.
- Inflammation in the filaments
  - Infiltration of any type of presumed inflammatory cells in the filament.

- Subepithelial inflammation

    o Infiltration of any type of presumed inflammatory cells in the subepithelial space of the lamellae together with presence of pale, yellow to brown, intracellular, granular pigment.