PHARMAQ
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FINAL REPORT
Risk factors, indicators and strategic management of gill disease in Atlantic salmon


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1. Oppsummering (Norwegian)

Nedsatt gjellehelse har blitt en viktig utfordring for norsk lakseoppdrett og fører til redusert fiskevelferd og økonomisk tap både i ferskvanns- og sjøvannsfasen av produksjonen. Det finnes både smittsomme og ikke-infeksiøse faktorer som påvirker utviklingen og konsekvensene av gjellesykdom. Gjellesykdom kan være en enkel eller sammensatt (kompleks) lidelse og utviklingen og konsekvensene av tilstanden avhenger av genetikk, ernæring, miljø og driftstekniske forhold. Det trengs mer kunnskap om hvilke risikofaktorer som bidrar til redusert gjellehelse, hvilke effekter notvasking og ikke-medikamentelle metoder for avlusning har på gjellehelsen, samt ytterligere indikatorer for å kartlegge redusert gjellehelse for å kunne utvikle strategier for å forebygge og redusere forekomsten av gjellesykdom, samt minimere konsekvensene av redusert gjellehelse når sykdom først har oppstått.

For å identifisere faktorer som kan påvirke gjellehelsen hos norsk oppdrettslaks ble det gjennomført en longitudinell studie der seksten grupper av laks ved åtte sjøanlegg og fra fire ulike settefiskanlegg ble fulgt med regelmessig prøvetaking gjennom det første året i sjø. Videre ble det gjort tre feltstudier for å undersøke om notvask og termisk og mekanisk avlusning kan ha akutte effekter på gjellehelsen hos oppdrettslaks og to pilotstudier for å undersøke om en håndholdt analyse-enhet for klinisk kjemi gir pålitelige resultater og om klinisk kjemi- og blodgass-analytter endres ved gjellesykdom.

Faktorer assosiert med gjellesykdom, gjellerelatert dødelighet og total dødelighet i ferskvannsfasen

Høyere vanntemperatur var assosiert med høyere total dødelighet i settefiskfasen, noe som betyr at det var høyere dødelighet ved høyere vanntemperatur. Det var ikke mulig å vurdere betydningen av miljøfaktorer eller infeksiøse agens for gjellehelsen i settefiskfasen da forekomst av gjellesykdom og gjellerelatert-dødelighet i prosjektperioden var for lav.

Faktorer assosiert med gjellesykdom, gjellerelatert dødelighet og total dødelighet i sjøfasen

Etter utsett i sjø var økende antall døgngrader i settefiskfasen forbundet med høyere total dødelighet de første 90 dager etter utsett, mens høyere vanntemperatur var forbundet med høyere total dødelighet både 90 og 180 dager etter utsett og i hele sjøfasen. I tillegg var makroskopisk gjellescore og avlusninger som involverte håndtering og/eller badebehandling forbundet med høyere total dødelighet. Miljø og helse i settefiskfasen og tidspunkt for sjøsetting hadde liten til ingen effekt på dødelighet og gjellehelse i denne studien.

Graden av gjellepatologi og antall registreringer av gjellerelatert-dødelighet i prosjektperioden var relativt lav. Resultatene under må derfor tolkes med forsiktighet. Gjellerelatert-dødelighet var høyere ved økende omfang av karskader i gjellene, men ingen andre mulige risikofaktor ble funnet i assosiasjonsanalysen. Omfanget av histopatologiske forandringer i gjelle var forbundet med høyere makroskopisk gjellescore, funn av amøber ved

histologisk undersøkelse og at fisken kom fra settefiskanlegg med RAS-system, og med lavere oksygennivå i sjøvannet. Alle fiskegruppene og anleggene fikk påvist *D. lepeophtherii* og *Ca.* B. cysticola-infeksjon i sjøfasen og infeksjonen vedvarte ut prosjektperioden. Det ble ikke funnet en sammenheng mellom disse organismene eller SGPV og gjellerelatert dødelighet eller graden av gjellepatologi i sjøfasen. SGPV og *N. perurans*-infeksjon i sjøfasen ble hovedsakelig påvist på sensommer og høst. Funn av patologiske forandringer typiske for amøbegjellesykdom sammenfalt med påvisning av amøber ved PCR og funn av amøber ved histologisk undersøkelse var forbundet med en økning i alvorlighetsgraden av gjellepatologi. Det ble ikke påvist skadelige algeoppblomstringer eller forekomst av skadelige nivåer av zooplankton ved hjelp av ukentlig eller sjeldnere uttak og analyse av vannprøver.

Akutt effekt av notvask og ikke-medikamentell avlusning på gjellehelsen

Ved undersøkelsen av akutte effekter av notvask og avlusning på gjellehelse ble det funnet en økning i antall fisk med subakutte karskader, hovedsakelig tromber, første dag etter notvask av nøter med moderat begroing. Eksponering for groemateriale ved notvask hadde liten og kortvarig effekt på gjellehelsen og denne effekten er trolig av liten klinisk betydning, men mer kunnskap trengs for å avgjøre eventuell betydning av notvask og eksponering for groemateriale for gjellehelsen.

Det var en økning i omfanget av karskader og lamellær hyperplasi i gjelle etter både mekanisk og termisk avlusning, men det var størst økning i karskader 24 timer etter mekanisk avlusning. Avlusing (med unntak av medisinfôr) var også forbundet med en økning i total dødelighet. Etter termisk avlusning såes en økning i antall fisk med gjellepatogener observert histologisk og en økning i mengde arvestoff påvist ved qPCR for *Ca.* B. cysticola. Disse resultatene viser at termisk og mekanisk avlusning har en negativ effekt på gjellehelsen og er forbundet med forøket dødelighet. Termisk avlusning kan føre til proliferasjon av mikroorganismen *Ca.* B. cysticola i gjellevevet. Det er uklart i hvilken grad de observerte gjelleforandringene og den økte forekomsten av mikroorganismer påvirker den kliniske tilstanden til fisken. Videre arbeid bør gjøres for å undersøke om funnet kan replikeres i et større materiale og om forandringene observert ved avlusning vil avheles eller om gjelleskader kan akkumuleres ved gjentatte behandlinger.

Klinisk kjemi for evaluering av gjellehelse og bruk av håndholdt blodprøvemaskin i felt

Den håndholdte analyseenheten iSTAT® gav ikke pålitelige målinger av nivået av ioner i blodet hos atlantisk laks. Det var godt samsvar mellom laktat-nivå målt med en iSTAT-maskin og en laboratoriemaskin. Milde makro- og mikroskopiske gjelleforandringer førte ikke til endringer i nivået av natrium, kalium, klorid, glukose og laktat i blodet til atlantisk laks.

2. Summary (English)

Reduced gill health has become an important challenge for Norwegian salmon producers and can lead to reduced fish welfare and economic losses in both the fresh water and sea water phases of production. Infectious and non-infectious factors can impact the development and outcomes of gill disease. Gill disease can be a simple or multifactorial (complex) condition, and the development and consequences of disease can depend on genetics, nutrition, environmental and managerial factors. More knowledge is needed to understand which risk factors contribute to reduced gill health, if in situ net cleaning and non-medicinal delousing has an impact on gill health, and to determine which are the most useful indicators of gill health status. This information can be used to develop strategies to prevent and reduce the prevalence of gill disease and minimize the negative consequences once disease has developed. To identify factors associated with gill disease in Norwegian farmed Atlantic salmon 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 first year at sea. Further, we did three field trials to examine if in situ net cleaning and thermal and mechanical delousing can have acute effects on gill health. Lastly, two pilot studies were performed to determine if a point-of-care device (POC-device) for clinical chemistry gives reliable results when used in Atlantic salmon, and if clinical chemistry and blood gas analyte levels is altered in fish with gill disease. In the freshwater phase we found that higher water temperatures were associated with higher overall mortality, meaning that more fish died at higher temperatures. It was not possible to assess the impact of environmental factors or infectious agents on gill health in the freshwater phase of production because the prevalence of gill disease, gill pathology and gill-related mortality during the project period was too low. In the sea phase of production an increasing number of daydegrees in the freshwater phase was associated with higher overall mortality the first 90 days after sea transfer. Higher water temperatures were associated with higher overall mortality both at 90 and 180 days after sea transfer and for the entire sea phase of production. In addition, increasing gross gill scores and delousing events including handling and/or bath treatments of the fish were associated with higher overall mortality. Environmental exposures and health status of fish during the freshwater phase had little to no impact on mortality or gill health in the current study. The extent of gill pathology and the number of recorded gill-related mortality cases in study period was relatively low. Because of this the results should be interpreted with caution. Gill-related mortality was increasing with increasing extent of vascular lesions in the gill, but no other factors was found to be associated with this outcome. An increasing extent of gill histopathology was associated with increasing gross gill scores, observation of amoeba in tissue sections, originating from a freshwater facility with RAS, and lower oxygen levels in the sea water. D. lepeophtherii and Ca. B. cysticola infections were detected in all fish-groups and sea water sites and infection persisted throughout the study period. There was no association between pathogen load of D. lepeophtherii, Ca. B. cysticola or SGPV and gill-related mortality or extent of gill histopathology in the current study. SGPV and N. perurans infections were predominantly found in late summer and autumn. Observation of gill lesions consistent with

amoebic gill disease coincided with detection of N. perurans by PCR, and observation of amoeba in tissue sections were associated with increasing extent of gill pathology. Harmful algal blooms or harmful levels of gelatinous zooplankton was not detected with weekly or less frequent sampling and analysis of sea water from the sea sites. Examining acute effects of in situ net cleaning and delousing on gill health we found an increase in the number of fish with subacute vascular lesions, primarily lamellar thrombi, the first day after in situ net cleaning of moderately fouled net-pens. The exposure to fouling material during net cleaning had a small and short-lived effect on gill health, and this effect is probably of little clinical significance, though more knowledge is required to conclusively determine if net cleaning and exposure to fouling material impacts gill health. Compared to pretreatment samples there was an increase in the extent of vascular lesions and lamellar epithelial hyperplasia in the gills after both thermal and mechanical delousing, but the increase was highest at 24 hours after mechanical delousing. Non-medicinal delousing and bath treatments were associated with an increase in overall mortality. An increase in the amount Ca. B. cysticola genetic material detected by PCR and an increase in the number of putative gill pathogens in tissue sections were seen after thermal delousing. These results suggests that thermal and mechanical delousing has a negative impact on gill health and are associated with increased mortality of treated fish. Thermal delousing may lead to proliferation of Ca. B. cysticola in the gill tissue. It remains unclear to what extent the observed gill lesions and increase in gill pathogens leads to clinical effects in affected fish. Further work is necessary to determine if the results are replicated in a larger material and whether the lesions observed after delousing can heal or if gill lesions can accumulate with repeated treatments. The POC-device iSTAT® did not provide reliable measurements of the concentration of selected ions in the blood of Atlantic salmon. A good agreement was found between lactate levels measured on the iSTAT® and a laboratory analyzer. Mild gross and microscopic gill pathology were not associated with changes in the blood or plasma levels of sodium, potassium, chloride, glucose, and lactate.

Main findings

Work package 1

- Increasing water temperature was associated with increased total mortality in the freshwater phase as well as with mortality for the time-periods up to 90 and 180 days after sea-transfer, and for the whole marine phase of production.
- Degree days in freshwater was associated with increased total mortality for the first 90 days after sea transfer, while none of the examined freshwater factors were associated with gill-related mortality or total mortality for the whole marine phase of production.

- The total mortality at sea was associated with increased water temperature, gross gill score and delousing involving bath treatment and/or handling of the fish.
- Gill-related mortality in the sea phase was only associated with increased severity of vascular lesions in the gill tissue.
- The severity of gill histopathology was associated with increasing gross gill score, observation of amoeba on histology and fish originating from a RAS freshwater site and with decreasing water oxygen levels.
- *D. lepeophtherii* and *Ca.* B. cysticola infection appeared to be ubiquitous in the sea phase of production in the assessed sites and these pathogens were not associated with gill related mortality or gill pathology in the current study.
- SGPV-infection during the seawater phase appeared to have a seasonal distribution with infection detected in late summer and fall. No association between SGPV-infection and gill-related mortality or severity of gill pathology was detected.
- There was a seasonal pattern in detection of *N. perurans* and lesions consistent with amoebic gill disease in late summer and fall, and observations of amoebae were associated with increased severity of gill pathology.
- The degree of gill pathology and the recorded gill-related mortality in the project groups were relatively low and due to this the above results should be interpreted with caution.

Work package 2

- The iSTAT POC-device was not demonstrated as a reliable tool for measurement of blood ions in Atlantic salmon.
- There is good agreement between the iSTAT and laboratory analyzer for lactate levels.
- Mild gill lesions are not associated with changes in blood sodium, chloride, potassium, or lactate.

Work package 3

- The number of fish with subacute vascular lesions (thrombi) in the gills at one day after *in situ* net cleaning was significantly increased compared to fish sampled before net cleaning.
- There was an increase in the number of vascular lesions and hyperplasia in the gills after both thermal and mechanical delousing.
- There was an increase in the number of fish with gill pathogens observed on histology and increased amount of genetic material of *Ca.* B. cysticola in gill tissue after thermal delousing.
- Results suggest a negative effect of thermal and mechanical delousing and net cleaning on gill health, but the clinical impact resulting from these lesions remains to be fully established.
- Further work should be undertaken to see if similar findings can be detected in a larger dataset and to determine if the observed lesions will resolve or can accumulate with repeated delousing or net cleaning operations.

Clinical implications

- The mechanical and thermal delousing methods examined were associated with an increase in gill pathology up to seven to eight days after treatment, showing that delousing has a negative effect on gill health. Delousing treatments (except medicinal feed treatments) were associated with increasing total mortality.
- An increased pathogen load of *Ca*. B. cysticola was found within 7 to 8 days after thermal delousing, though the clinical significance of this increase is unclear. However, this suggest that thermal delousing is a stressor that can allow for increased proliferation of microorganisms in the gills.
- Little to no clinical impact of net cleaning on gill health and mortality could be found in the current study, however, significant knowledge gaps remain.
- There were not found any or only a small effect of timing of sea transfer and freshwater treatment system on mortality and gill health during the sea phase.
- There were no harmful algal blooms detected at these sites during the study period through weekly or less frequent samples. However, sampling should be considered on a site-by-site basis considering high risk periods, site, and water body history.
- The total gross gill score was positively associated with total mortality suggesting that total gross gill score could be of value for health monitoring, however the low gross gill scores and relatively mild pathology detected during the project period made it difficult to evaluate total scores usefulness.

Knowledge gaps

- RAS and flowthrough
    - The potential effects of different water treatment system on gill health needs to be further examined.
- Plankton
    - What are the plankton levels necessary to cause disease and harm?
    - What are the factors contributing to the importance of plankton and which environmental interactions are causing harmful effects on fish and increasing the pathogenic potential of plankton (toxin production, oxygen consumption)?
    - To what extent does zooplankton (Cnidarians) contribute to development of gill disease in Norway?
    - Can plankton exposure cause subclinical effects making fish more vulnerable to other gill pathogens or cause cumulative damage?
    - What are the best and most cost-effective method of plankton monitoring for fish farms?
- Delousing

    - What is the mechanism of gill damage detected in association with thermal and mechanical delousing?
    - Will the detected gill lesions repair and resolve and how long does it take for lesions to heal?
    - Can repeated delousing lead to cumulative gill damage?
    - What are the causes and mechanisms of treatment related mortality?
    - Will outcomes in terms of gill damage and mortality be worse if gill pathology is present when treatment starts?
- Net cleaning
    - Can repeated cycles of net cleaning lead to gill damage and if so, can the effect be cumulative?
    - What is the impact of the different biofouling organisms and the composition and life stage of the biofouling community at the time of net cleaning?
    - What is the impact of differences in biofouling level, biofouling strategies, site-specific factors, health status and size of the fish at time of net cleaning?
- Gill pathogens
    - Further work is necessary to determine the significance of *Ca*. B. cysticola and *D. lepeophtherii* as gill pathogens in sea farmed salmon.
    - The significance of SGP-virus as a gill pathogen during the sea phase is unclear
    - The interactions between pathogens and environmental factors and management operations needs further study.
- Gill indicators
    - The usefulness of the total gross gill score used in the project needs to be further assessed.

3. Introduction

Compromised gill health has emerged to be a major factor in the farming of salmonids in Norway and across the world, leading to economic losses and reduced animal welfare in both the marine and freshwater phase of production. In 2020 multifactorial gill disease was considered as one of the most important causes of mortality in the Norwegian ongrowing salmon farms (Sommerset, et al., 2021). During the production cycle salmon are exposed to multiple challenges that can lead to reduced gill health, including both infectious diseases and environmental conditions. Some problems may be common to both the freshwater and seawater stage, but each life stage also presents unique challenges connected to their various production conditions.

The introduction of new technologies in the freshwater stage may also have contributed to gill problems related to water quality in the salmon hatcheries. Recirculating aquaculture systems (RAS) have become increasingly common in land based smolt production in Norway. However, with the increased use of RAS, as opposed to flow-through (FT) systems, there have been observations of increased health problems such as nephrocalcinosis. The mechanisms behind this are currently being investigated, but high stocking densities and accumulation of CO_2 can play an important role (Fivelstad, et al., 2018; Fivelstad, et al., 2003; Mota, et al., 2019). S0 smolts are transferred to the sea in the autumn, under a decreasing photoperiod, while larger 1+ smolts (S1s) are sea transferred in the spring. Spring transferred smolt (S1) were found to have higher losses compared to the fall generation (S0) when studying 318 out of a total of 402 production sites in operation in Norway from fall 2010 and through 2011 (Pincinato, et al., 2021). Previous studies also support this finding and concluded with better survival in S0 versus S1 smolts (Kristensen, et al., 2012; Lysfjord, et al., 2004).

Common infectious gill pathogens in Norwegian salmon production include *Neoparamoeba perurans* (syn. *Paramoeba perurans*), *Ca*. Branchiomonas cysticola, salmon gill poxvirus (SGPV) and *Desmozoon lepeophtherii* (syn. *Paranucleospora theridion*). *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 (Sommerset, et al., 2021). Epitheliocystis is a term used to describe disease associated with the presence of intracytoplasmic bacterial cysts (epitheliocysts) in the gill and skin epithelium of different fish species. There are several agents associated with the presence of gill epitheliocysts in Atlantic salmon, including *Ca*. Branchiomonas cysticola described both in fresh and seawater (Mitchell, et al., 2013; Toenshoff, et al., 2012; Wiik-Nielsen, et al., 2017). Salmon gill poxvirus (SGPV) is mainly associated with gill disease in the freshwater phase although the virus is commonly detected through the entire production cycle (Gjessing, et al., 2017; Gjessing, et al., 2015). The microsporidian *D. lepeophtherii* has a complex life cycle and infects both sea lice (*Lepeophtheirus salmonis)* and Atlantic salmon (Freeman and Sommerville, 2011; Nylund, et al., 2010). The parasite can be detected in seawater all year round, but higher levels of the parasite are found in autumn. It is often detected in healthy fish but is detected in higher densities in fish suffering from gill disease (Steinum, et al., 2010). Other parasites, fungi and

bacteria may also infect the gills, including *Tenacibaculum* spp. (Mitchell, et al., 2011) and in recent years *Pasteurella* spp. (Legård and Strøm, 2020; Valheim, et al., 2000). Co-infections involving several of the above-mentioned pathogens are common and a challenge for the management of gill diseases (Downes, et al., 2018; Gjessing, et al., 2019; Gunnarsson, et al., 2017; Herrero, et al., 2018).

Non-infectious gill disease such as that attributed to harmful algal blooms (HAB) and jellyfish swarms are a significant problem for global salmon mariculture (Baxter, et al., 2011). During the spring 2019 acute HAB associated to the species *Chrysochromulina leadbeaterii* was causing severe mortality in salmon farms in Northern Norway (Karlsen, et al., 2019). Approximately 8 million salmon died during the bloom and the cost was estimated to be 2.3 to 2.8 Billion NOK (Marthinussen, et al., 2020). In Ireland, on site monitoring of potentially harmful zooplankton and phytoplankton species, using plankton nets and light microscopy, is considered a key tool for a complete diagnosis and vital to provide feedback to the industry and management advice for the mitigation of gill disease. On site plankton monitoring is not common in Norwegian fish farming, while some companies in Scotland monitor farms in areas with risk of development of plankton blooms.

Biofouling of net pens is a major problem potentially causing reduced waterflow in pens, leading to reduced oxygen levels and water quality, which in turn can affect gill health. It has been suggested that stress induced by net cleaning (Stene, et al., 2018) could trigger disease outbreaks (Bloecher and Floerl, 2020). Experimental exposure of fish to hydroids indicate that net washing can lead to gill pathology and reduced gill health (Baxter, et al., 2012; Bloecher, et al., 2018), but whether fish develop acute gill lesions and decreased gill health after net washing in the field remains unknown. Napsøy (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.

During the production cycle salmon also go through treatments and handling that can affect gill health. Concerns have been raised about potential negative effects on fish and gill health during and after thermal and mechanical sea lice treatments (Hjeltnes, et al., 2018; Poppe, et al., 2018). A range of injuries involving skin, fins, gills, heart, cranium and brain have reported after thermal and/or mechanical delousing (Bentzen, et al., 2018; Erikson, et al., 2018; Gismervik, et al., 2017; Hjeltnes, et al., 2018; Jørgensen and Rød, 2019; Nilsen, et al., 2010; Poppe, et al., 2021; Poppe, et al., 2018; Sommerset, et al., 2021), though 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. Laboratory experiments (Moltumyr, et al., 2021) suggests that the treatment component of thermal delousing (i.e., warm water exposure for 30 seconds) does not directly cause acute injuries, but this does not exclude the possibility of injuries and reduced health due to stress and behavioural responses during treatment. Most the of initial studies examining the effect of non-medicinal delousing

on salmon included relatively few test replicates, limited follow-up time and involved a single treatment of the fish. In addition, gross examination and scoring of skin, eye, gill and fin lesions, and percent mortality after treatment were the main tools used to assess the effect of delousing on the treated fish. As such subtle, indirect, cumulative and long-term effects on overall health and gill health specifically were unlikely to be detected and the impact of these treatments on short-term and long-term health outcomes remains inadequately documented (Moltumyr, et al., 2021).

The aim of the present project has been to generate knowledge and develop tools that can contribute to preventing mortality and welfare challenges associated with reduced gill health and gill diseases in farmed Atlantic salmon by identifying risk factors and indicators of gill disease.

4. Project organization and objectives

Organization

The GillRisk project is an answer to the FHF «open call»: "Knowledge and tools that can prevent mortality and welfare challenges associated with reduced gill health and gill diseases in farmed salmon fish". The GillRisk project consortium consisted of Fish Vet Group Norge (now Pharmaq Analytiq AS), Mowi ASA, VAI Consulting and Norwegian University of Life Sciences (NMBU).

The project was led by FVG Norge now a part of Pharmaq Analytiq, with Marta Alarcón as project leader and Liv Østevik conducting her PhD within the project. Marta Alarcón, Marianne Kraugerud and Liv Østevik oversaw WP1, 2 and 3, respectively. Other researchers at former FVG (Kai-Inge Lie and Hege Hellberg) were involved in histopathology analyses and in drafting reports and manuscripts for publication. Mowi represented by Farah Manji, Benedicte Simensen and Gordon Ritchie was the industry partner. Mowi provided access to the sites and conducted field sampling. They were also responsible for identification and traceability of stock groups, gathering of farm and pen data (stocks, treatments, mortalities, gill scores, etc.) and supplying other relevant production data. Måsøval AS, represented by Andreas Skagøy, was an additional industry partner supplying production data, sites, and fish for two studies conducted as a part of WP3. Ane Nødtvedt and Marit Stormoen, from NMBU, were responsible for epidemiological and statistical analysis in the project. Hamish Rodger from VAI Consulting has served as scientific advisor and was involved in histopathology analysis.

The reference group consisted of Andreas Skagøy (Måsøval), Henrik Duesund (Cermaq) and Geir Schriwer (Salmar). Sven M. Jørgensen (FHF) has participated in the meetings with the reference group.

Objectives

- Identify infectious and non-infectious risk factors that contribute to the development of gill disease and reduced gill function as measured by standardized gross and histologic gill scoring and identify the relative importance of the different factors and their interactions in freshwater and seawater. (Work package 1)
- Identify operative indicators of reduced gill health and function to be applied in management decision-making at farm level. (Work package 1 and 2)
    - Establish and validate on farm diagnostic methods for gill disease to prevent or minimize its impact.
    - Establish and assess the usefulness of zooplankton and phytoplankton monitoring on Norwegian sea farms.
    - Validate portable blood physiology point-of-care (POC) devices for farmed Atlantic salmon, as non-lethal and immediate tools to be used before management operations and to improve on-site diagnostics.
- Provide information on short-term and long-term effects of selected stressful management operations on gill health. Document potential effect of reduced gill health on the outcome (mortality rate) of these operations. (Work package 1 and 3)
- Use results of statistical and epidemiological analysis to develop a statistical model and a prototype web-based calculator (application) in which data from water monitoring and fish health screening are used as input to calculate risk associated with stressful management procedures (delousing, transportation etc.). (Work package 1 and 3)

5. WP1 – Risk factors and development of gill disease throughout the production cycle

4.1 Materials and methods

4.1.1 Study population and sites

Four freshwater sites and eight seawater sites were included in the project (Figure 1). Freshwater sites were in production areas (produksjonsområde (PO)) 5 and 6. Two freshwater sites used flow through systems (FT) (site 1 and 4), one used recirculating aquaculture systems (RAS) (site 3) and one site used both RAS and FT systems (site 2). A winter signal was given at site 1 (12 hours light:12 hours dark for 5 weeks, S0 fish only) and 3 (12 hours light:12 hours dark for 6 weeks), and salinity was increased prior to sea transfer to facilitate smoltification at sites 2-4. Freshwater sites were selected to include two sites with a history of gill disease (site 3 and 4) and two sites without known gill issues (site 1 and 2). From each freshwater site a group sea transferred at autumn 2018 (S0) and a group sea transferred in spring 2019 (S1) was included. After sea transfer the project groups were split in two pens at same sea site, resulting in 8 separate project groups during the freshwater phase and 16 project groups during the sea

water phase of production (Fig. 1). Sea sites were in PO4, PO5 and PO6 and the number of stocked pens per farm ranged from 6 to16. Four sea sites had a history of problems with gill related mortality (site A, C, D and G), while the other sites only had mild or no recorded gill related mortality.

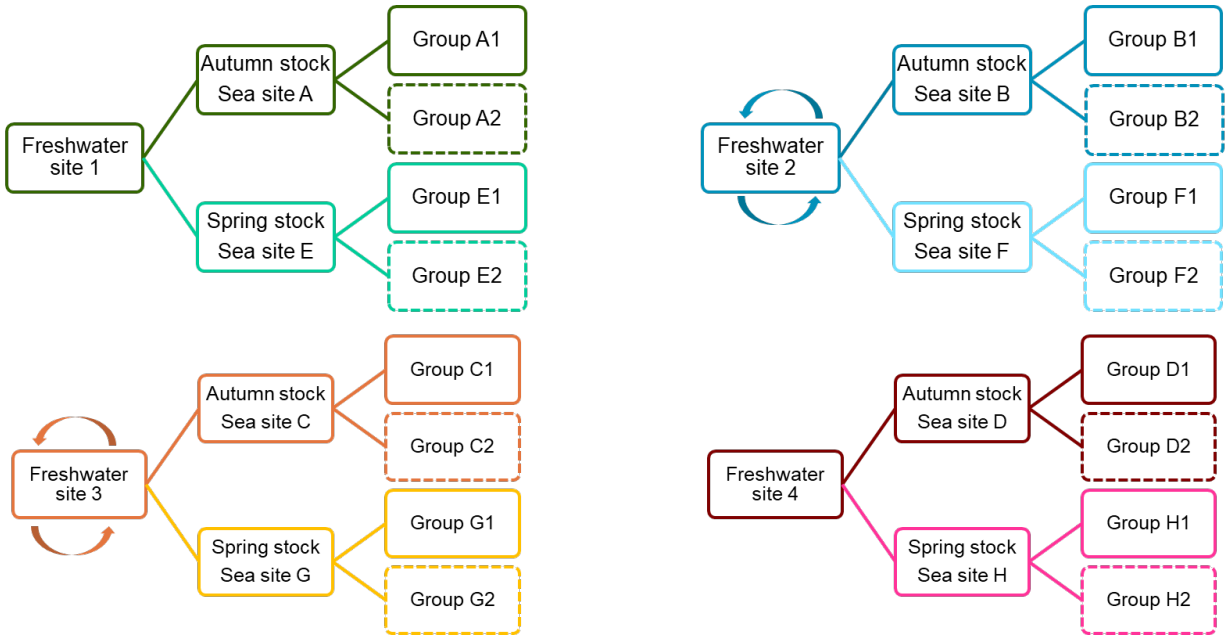


Figure 1. Overview of included freshwater sites (n=4), sea sites (n=8) and pens (n=16) in a longitudinal study of gill disease in farmed Atlantic salmon in Norway. Stocking period (S0=autumn stock/S1=spring stock) and water treatment (flow-through vs. RAS=circular arrow) in indicated. Color coding of fish groups is consistent throughout presentation.

Most sites used nylon nets with copper dioxide coating and washed nets, while one site (site B) used environmental nets. Fish groups were subjected to net cleaning and delousing operations as outlined in Table 1 and Fig. 2.

Fish group	Delousing operations*	Net cleaning site	Net cleaning pen	Nettype
A1	1*	2	1	Aquanet Ultra/Netwax A7 microfino
A2	5*‡	2	0	Netwax A7 microfino
B1	5	3	0	Environmental nets
B2	5	3	0	Environmental nets
C1	11*	3	1	Netwax NI3
C2	10*	3	2	Aquanet Boost
D1	7	11	11	Aquanet Ultra/Netkem, Netpolish Np Super
D2	8	11	11	Aquanet Ultra/Netkem, Netpolish Np Super
E1	8	0	0	Aquanet Boost/Netkem, Netwax A7 Microfino
E2	7	0	0	Aquanet Boost/Netkem, Netwax A7 Microfino
F1	3	0	0	Aquanet Boost/Netkem, Netwax A7 Microfino
F2	2	0	0	Aquanet Boost/Netkem, Netwax A7 Microfino
G1	0	2	0	Aquanet Boost/Netkem, Netwax E5 Greenline
G2	1	2	0	Aquanet Boost/Netkem, Netwax E5 Greenline
H1	8	0	0	Aquanet Boost/Aquanet Ultra
H2	8	0	0	Aquanet Boost/Aquanet Ultra

Table 1. Number of delousing operations and *in situ* net washing per fish-group, not including in-feed treatments. *Groups A and C were the only groups not receiving in-feed delousing treatments. ‡ One treatment of group A2 was a combined freshwater and thermal treatment.

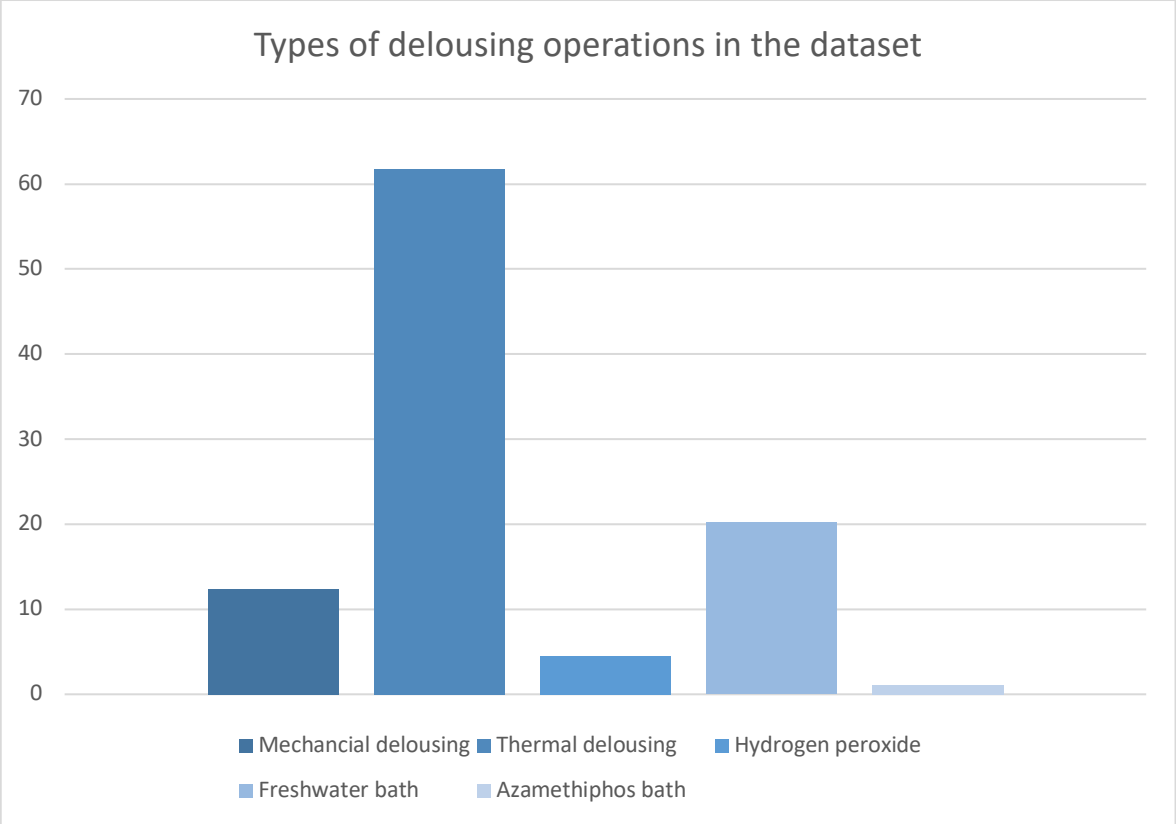


Figure 2. Frequency of the different delousing methods used in the project sites, excluding in-feed treatments.

4.1.2 Sampling

In the freshwater phase 20 to 30 fish per project group were sampled 0 to 3 times. During the first year at sea, ten to thirty fish per pen were sampled every four to six weeks (see Appendix 1 overview of samples). The plan was to sample up to 15 fish with clinical signs of gill disease and 15 presumed healthy fish per pen, but most fish sampled showed no clinical signs of gill disease and were presumed healthy or of unknown health status. The 2nd gill arch on the left side was sampled for histology, while the 3rd left gill arch was sampled for qPCR. Gross gill scoring (n = 20 fish per pen) was performed weekly during the sea phase. When a project pen was sampled for histology and qPCR gross scoring was most often performed on the sampled fish so that parallel gross scores, qPCR, and histology results were recorded. Due to bad weather and restrictions associated with Covid-19, there were some deviations from the sampling and gross scoring plan. In addition, no samples were collected from the freshwater phase for project groups B and C as these fish already were transferred to the sea sites when the project sampling was initiated.

4.1.3 Water samples – plankton analysis

Collection of sea water for plankton analysis was conducted weekly and analysed weekly to monthly. The plankton protocol was adapted from a system developed by Fish Vet Group UK and the site staff received a training session. Plankton was collected using 250 µm mesh nets, with 25 cm and 50 cm diameter ring for phyto- and zooplankton, respectively (for sampling protocol see Appendix 2). The nets were submerged to 10-meter depth and slowly pulled towards the surface. Once on the surface, water was poured down the outside of the net to further concentrate the sample into the filter. Approximately 300 ml of seawater was used to wash the contents of the filter into the phytoplankton collecting bottle and 5 ml Lugols iodine solution was then added for preservation. The zooplankton samples were immediately fixed in formalin (4%) in 50% solution with sea water. The samples were stored avoiding direct sunlight and at cool temperatures before sending to the laboratory.

In the lab, a small subsample of the phytoplankton (1 ml) was placed into a Sedgewick counting chamber slide for assessment under an inverted microscope. The number of squares counted depended on the density of organisms in the sample: 0-5 cells/ square (50 squares), 5-10 cells/ square (25 squares), 10-20 cells/ square (12 squares), 20-50 cells/ square (10 squares). The sample was diluted if there were more than 50 cells/ square. The number of algal cells per liter water was calculated, and the number of different species grouped as: unspined diatoms, *Chaetoceros* spp., *Pseudo-nitzia* spp., *Ceratium* spp., *Rhizosolenia* spp. and others (Fig. 7). To calculate the number of cells per liter the following formula was used:

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

The sample concentration factor was calculated as follows:

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

For zooplankton quantification a subsample of 150 ml was transferred to cell culture bottles for evaluation under the stereomicroscope. All organisms in the subsample that were considered potential harmful to fish were counted and the number of zooplankton per cubic meter of seawater was estimated.

To calculate the number of zooplankton per cubic meter the following formula was used:

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

4.1.4 Gross gill score

A gross gill system based on the total area of abnormal tissue in the gill was adapted from a system developed by Fish Vet Group UK (see Appendix 3). Each left gill arch was scored separately on a scale from 0 - 5 and then a mean and sum gill score for all arches were calculated. Lesions counting towards the scored included:

- White areas and white patches/plaques (presumed hyperplasia)
- Haemorrhages
- Loss of gill tissue
- Swollen, thickened gill tissue
- Yellow discoloration of gill tissue
- Fusion of filaments
- Necrosis (defined as grey discolored tissue)

See figure 3 for example images of gross lesions. In addition, scorers were asked to record, but not score diffusely red gills (presumed hyperemia), diffusely pale gills (possible anemia), injuries or deformities of the operculum, injuries on gill arch, increased amount of mucus on the gills and signs of respiratory distress. The score categories were as follows:

- 0 – no abnormal gill tissue
- 1 – <5% of gill tissue with any lesion
- 2 – 5 to 25% of gill tissue with any lesion
- 3 – 25 to 50% of gill tissue with any lesion
- 4 – 50 to 75% of gill tissue with any lesion
- 5 – 75 to 100% of gill tissue with any lesion

Scorers included site staff and attending fish health personnel and veterinarians and varied from site to site and over time. At the start of the project site staff received a training session where the score system was explained and images of the different types of lesions and scores was demonstrated.

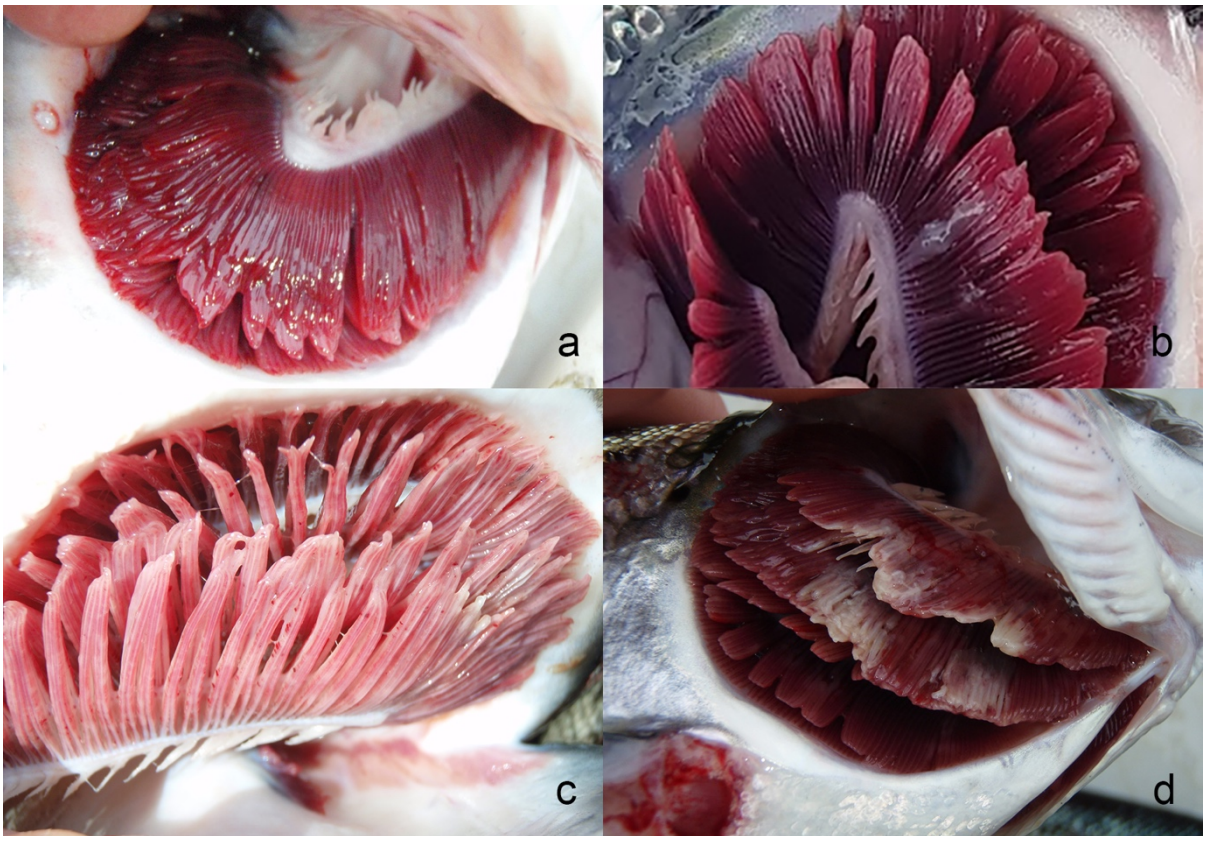


Figure 3. Gross pathology. a) Normal gill tissue, b) Hyperplasia, evident as white raised mucoid areas on the gill surface, c) Haemorrhage, multifocal red spots in the gill. Also note diffuse pallor and strings of mucus between gill filaments, d) Necrosis and tissue proliferation, evident as loss of tissue at the tips of filaments and as white discoloration of filaments. Image's courtesy of Hamish Rodger (a, c, and d) and Marianne Elnæs (b).

4.1.5 Histology

Gills were fixed in formalin and routinely processed, sectioned, and stained with hematoxylin & eosin (HE). Stained slides were scanned and read using a slide reading software. All counting and measurements were done using the annotation tools in the slide reading software. For WP1 the same pathologist (LØ) read all samples collected during the sea phase, while two different pathologists (HH and MA) read the gill samples from the freshwater phase. The pathologists were "blinded" with regards to qPCR-results, gross gill scores and results of water sample analysis.

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

- Vascular lesions (aneurysms and thrombi)

- Lamellar epithelial hyperplasia (including hyperplasia and hyperplastic and inflammatory lesions)
- Necrosis (of at least 1 lamella)

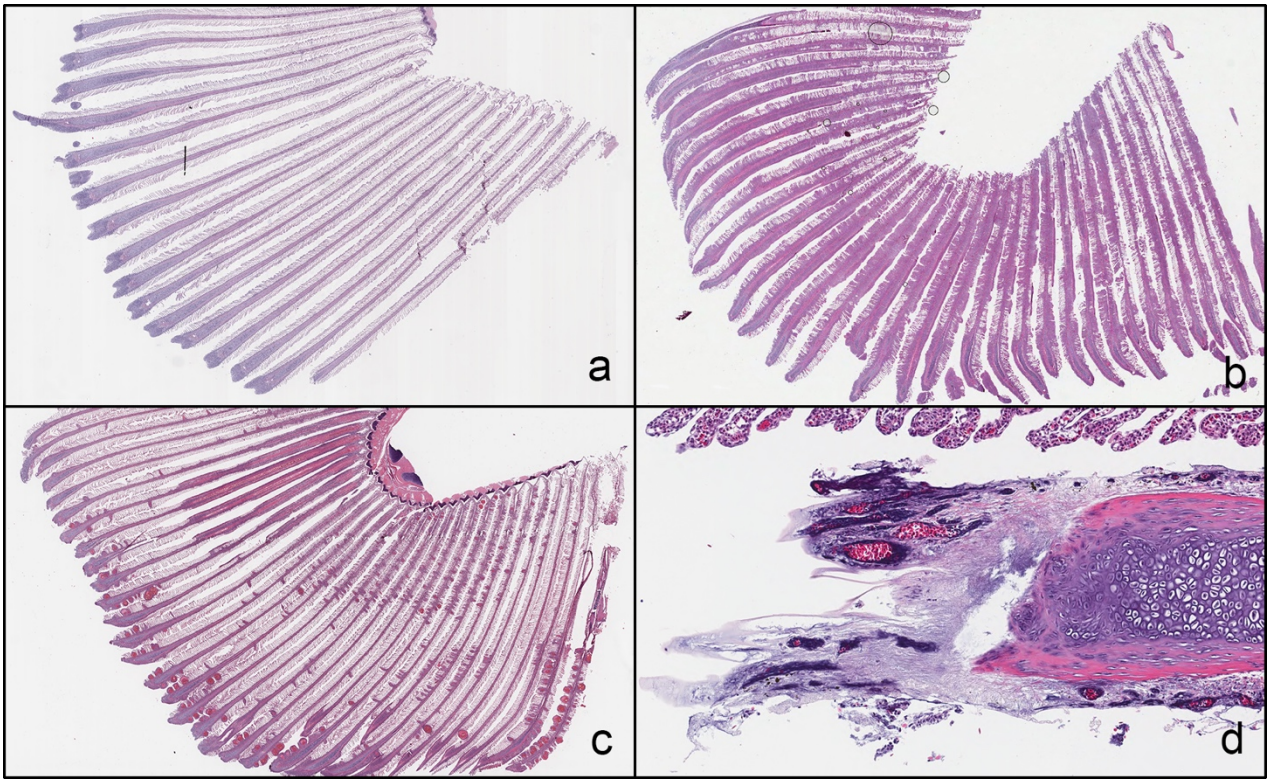


Figure 4. Histopathology, lesions recorded as counts and percent. a) normal tissue, b) multifocal hyperplasia of gill tissue, c) multifocal vascular lesions (aneurysms), d) focal necrosis of a filament tip. Note loss of normal tissue structures and large amounts of filamentous bacteria likely *Tenacibaculum* sp. in the necrotic tissue.

For WP1 a total histology count was calculated by summarizing the number of lamellae with the lesions listed above and subtracting the number of lamellae with two or more lesions at the same time. The total histology percent was then calculated by dividing the total histology count by the estimated lamellar count and multiplying by a 100. Any pathogens or organisms observed in or associated with the gill tissue were also recorded as present or absent. For statistical analysis in WP1 the median total percent tissue affected (referred to as median percent total histology score) per pen and sample point was used. For lesions and pathogens recorded as dichotomous variables the percent fish with a given lesion or pathogen per pen/sampling point was used.

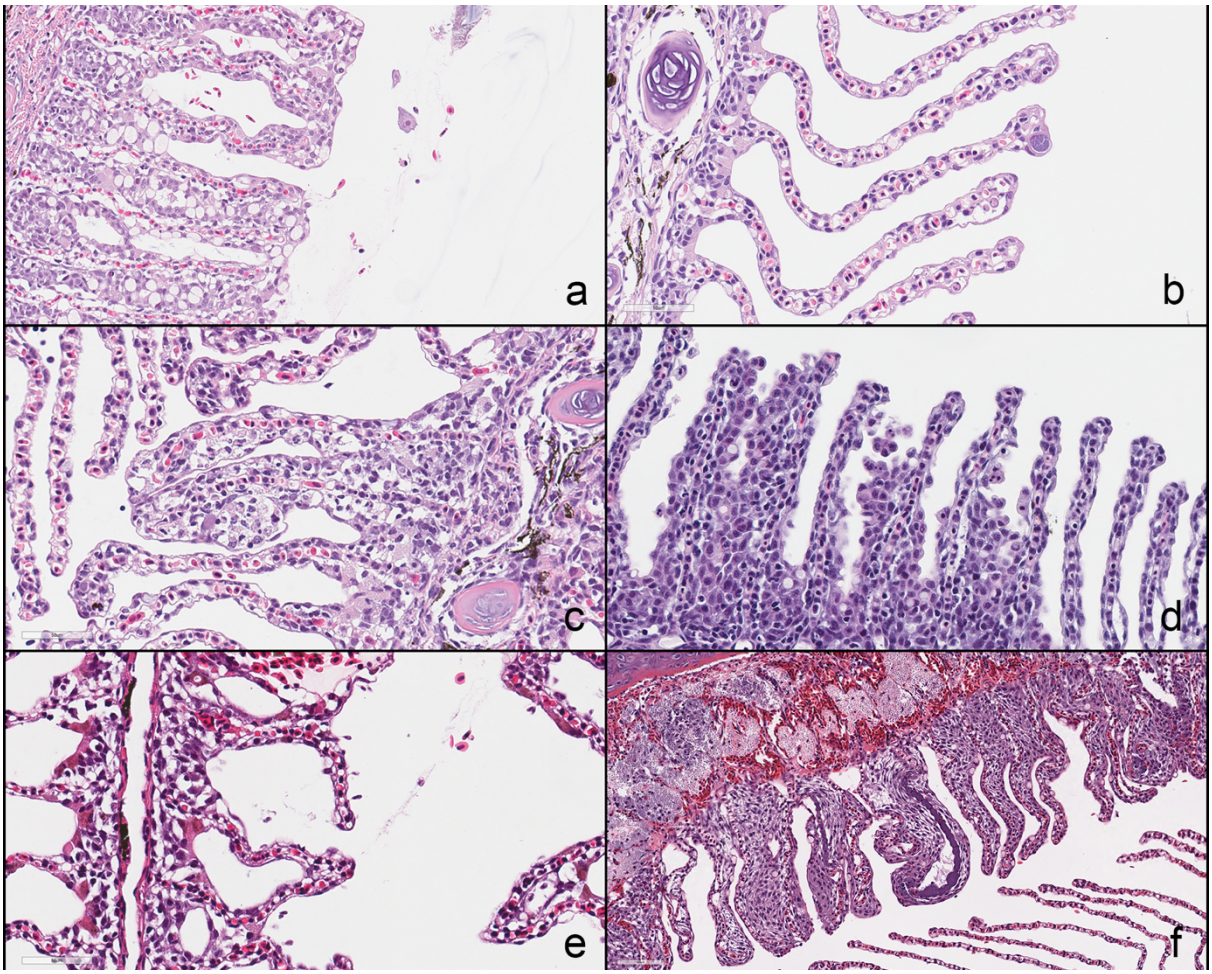


Figure 5. Histopathology, pathogens, and lesions associated with infectious agents. a) Lamellar epithelial hyperplasia and an amoeba (most likely *N. perurans*). Also, note mucous cell hyperplasia. b) Epitheliocyst, intracellular bacteria c) Epitheliocysts and subepithelial inflammation with degeneration of inflammatory infiltrates. Subepithelial inflammation and necrosis has been associated with *D. lepeophtherii*-infection but is probably not specific for this pathogen. d) Lamellar epithelial hyperplasia and epithelial apoptosis are lesions associated with SGPV-infection, e) *Ichthyobodo* sp. ("costia") and lamellar adhesions, f) Bacteria, haemorrhage, and lamellar epithelial hyperplasia. Note basophilic granular material (bacteria) partially embedded in eosinophilic material (fibrin) expanding filament vessels and lamellar sinusoids. *Pasteurella* sp.-infection was confirmed at the site.

4.1.6 qPCR analysis

Gill tissue from the third left gill arch were used for RT-qPCR for gill pathogens. Samples from the freshwater phase were analyzed for pathogens *Candidatus* Branchiomonas cysticola and salmon gill poxvirus (SGPV), while gill tissue collected during sea phase were analyzed for *Candidatus* Branchiomonas cysticola*, Paranucleospora theridion/Desmozoon lepeoptheirii*, salmon gill poxvirus (SGPV) and *Neoparamoeba perurans*. Reverse Ct-values were calculated using the following formula: reverse Ct = 40 – raw Ct-values. Reverse Ct-values of 40 were set

to zero. For calculation of mean Ct-values and normalized Ct-values, the negative Ct-values were set to 45. The normalized Ct-values were then calculated as follows:

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

4.1.7 Productional data and environmental data

Production data, health data from routine diagnostic sampling or screening and environmental registrations from all sites were retrieved through the management database Mercatus Farmer (ScaleAQ, Norway). Information was transferred from the central database owned by Mowi. Data-cleaning was performed, and data merged by fish-group to combine histology, plankton, PCR results and gross gill scores with the production data.

4.1.8 Statistical analysis

The software package Stata 15 (Stata Corporation) was used for all statistical analysis. Descriptive data are presented as plots of total mortality, gill related mortality, vascular lesions and more. Handling, diagnostic tests and other events were plotted by day of occurrence. Because all observations were clustered by site and fish-group over time, mixed models were used in the statistical analysis. First, potential factors were screened in univariable models controlling for site and fish-group. A liberal cut-off for statistical significance ($p<0.2$) was applied in the screening process. Subsequently, multivariable models were built to assess the association between several potential risk factors while still controlling for fish-group and site. Two sub-models (fresh- and seawater) were constructed for overall mortality outcomes and any significant variables were then combined in a final model. For the final models, the cut-off for statistical significance was set to $p<0.05$. Two main regression models were used:

- Linear mixed model regression for total mortality and total histology 14 days (all log transformed)
- Negative binomial mixed model for number of fish dead to gill related cause/day

The assumption of normally distributed residuals was assessed by plotting standardized residuals against inverse normal values in a normal-quantile plot.

4.2 Results

4.2.1 Water samples – plankton data

We analysed 326 plankton samples (phyto- and zooplankton) from Autumn 2018 to Summer 2020. The number of samples varied from 29 to 57 sampling points per sea site. The levels of plankton in the project sites were considered low with highest levels of phytoplankton during

spring and summer (Table 2 and Fig. 6). Based on the submitted samples there were no significant phyto- or zooplankton blooms during the study. While there were some variations in the phytoplankton levels, zooplankton levels were generally low throughout the sampling period. Project sites did not report of problems related to algae or jellyfish during the sea phase at any of the farms. No increase in gross or histologic gill scores or gill related mortality was evident following the peaks in total phytoplankton levels at site C and D, and there was no clear co-variation between phytoplankton levels and any of our gill indicators. Due to this, plankton levels were not included in further analysis for risk factors of gill disease.

Sites	Sampling period	N samplings	Perid of max level of plankton	Max plankton level (cells per liter)	Unspinned diatoms (cells per liter)	*Chaetoceros* spp (cells per liter)	*Pseudonitzia* spp (cells per liter)	*Ceratium* spp (cells per liter)	*Rhizosolenia* spp and others (cells per liter)
A	Nov 18 - Dec 19	38	Spring 2019 (May-June)	57385	25179	34407	6234	611	30681
B	Oct 18 - Dec 19	31	Spring 2019 (April-May)	47095	32607	36914	1809	146.7	6342
C	Nov 18 - Dec 19	33	Spring 2019 (March)	732164	461422	156456	122231	269	29948
D	Nov 18 - Jan 20	36	Spring 2019 (March)	535779	387744	12223	148035	476.7	2911
E	Aug 19 - Aug 20	27	Summer 2019 (Aug)	18896	11123	5500	2762.4	97.8	366.7
F	Jul 19- Oct 20	45	Summer 2019 (Aug)	59160	44369	11000	11489	158.9	1222.3
G	April 19 -June 20	57	Spring 2020 (March)	231016	223682	24568	28235	342	2090
H	April 19- June 20	55	Spring 2020 (March)	311179	306086.8	82862	161345	1222.3	24507

Table 2. Max level of phytoplankton (cells per liter) per site.

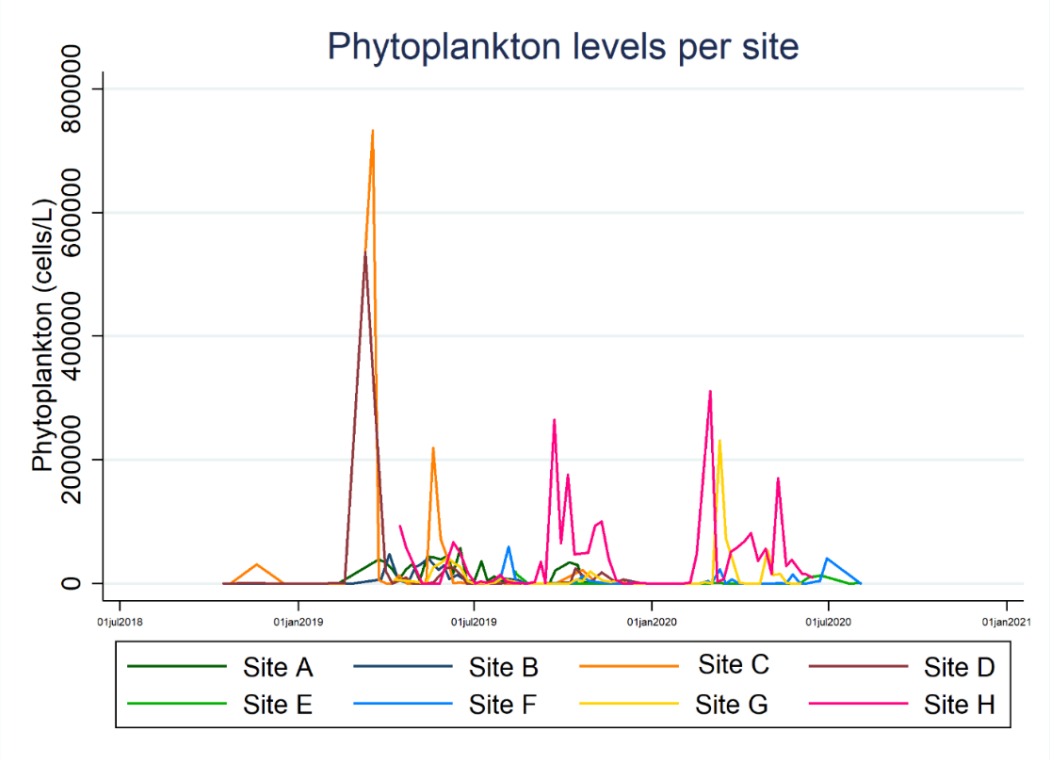


Figure 6. Phytoplankton level (total cells per liter) per site during the study period.

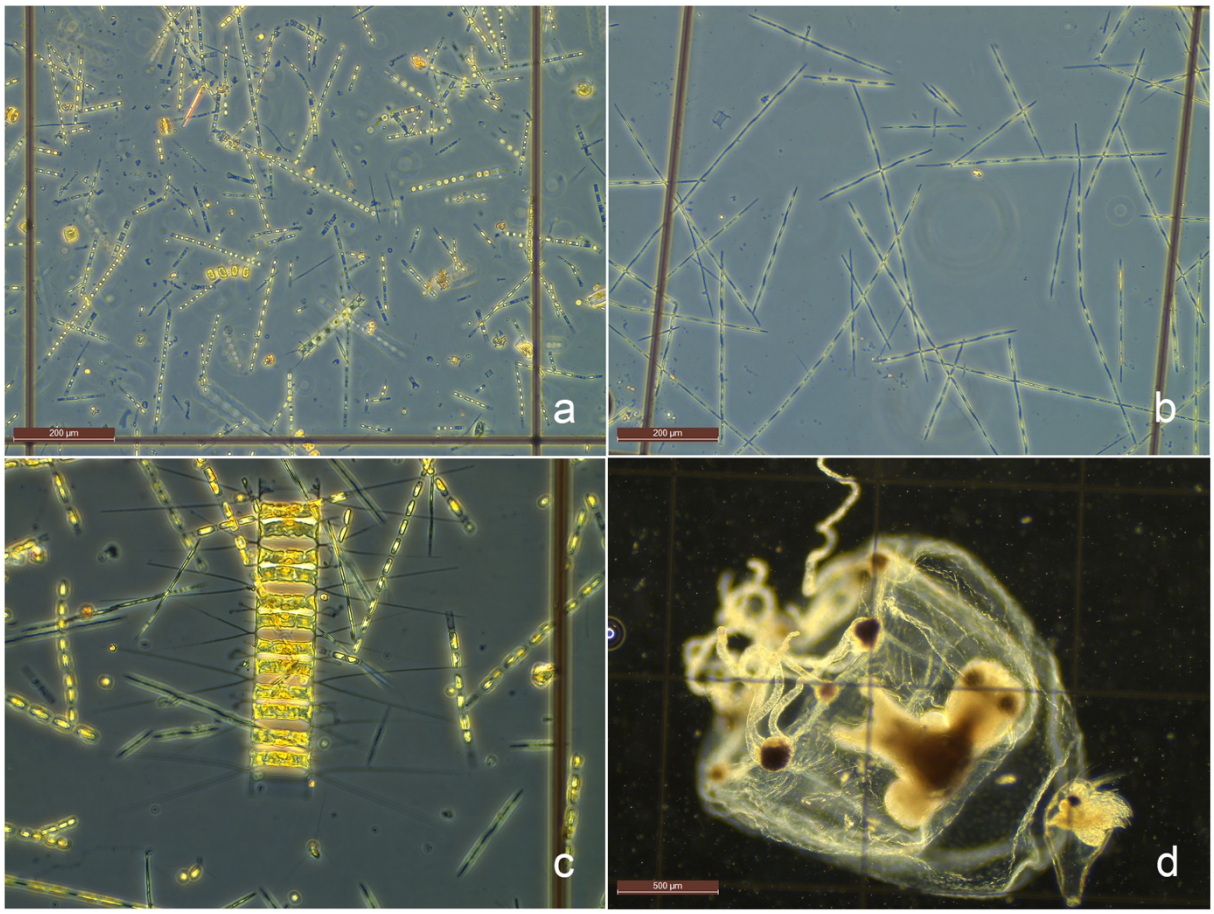
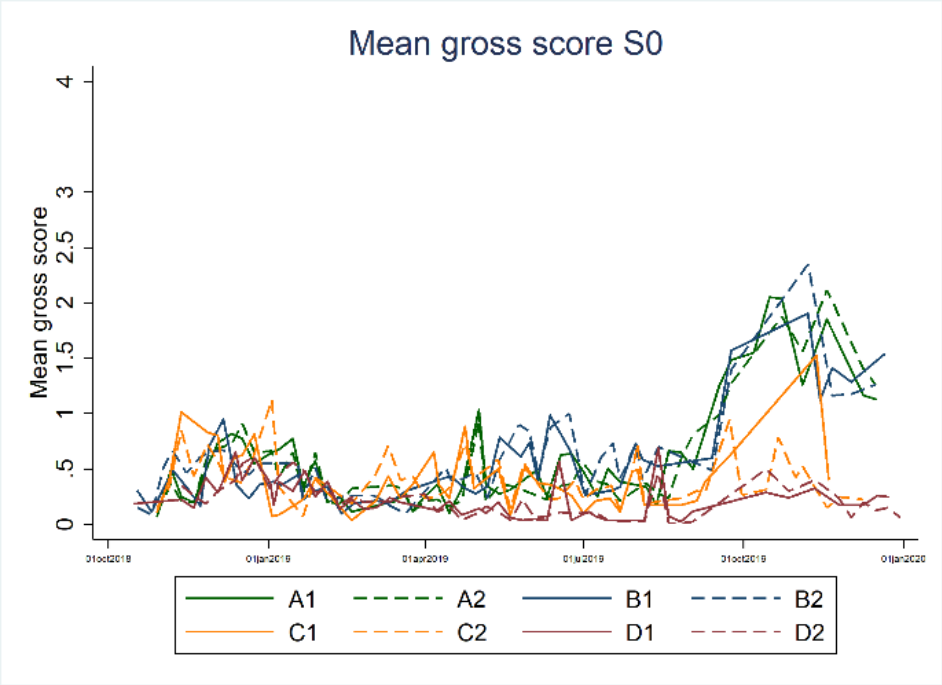


Figure 7. Plankton. Representative plankton species identified during the present study. a) unspined diatoms b) *Pseudo-nitzschia* spp. c) *Chaetoceros* spp. and d) microscopic jellyfish (*Cnidaria*), suspected *Rathkea* sp.

4.2.2 Gross gill scores

Mean gross gill scores per group and time point were generally low, mostly below 1 (range 0-5) (Fig. 8) and did not appear to show a clear seasonal variation or consistent co-variation with gill-related mortality, total histology-scores or prevalence of the putative gill pathogens detected by qPCR-analysis. An increase in gill scores was observed towards the end of the production for S0 groups, notably in sites A and B which also experienced gill-related mortality and increasing histology gill scores. However, no increase in gross gill scores was evident during the time period when sea site G experienced gill-related mortality, increased total histology gill scores and a high prevalence of *N. perurans*. Further, when plotting gross gill scores of the 2nd left gill arch against total histology scores (data not shown) increased histology scores were not clearly reflected in increased gross scores. Due to the relatively poor agreement between gross scores and the other two gill outcomes, and the overall low gross gill scores risk factor analysis using gross gill score as an outcome was not prioritized.



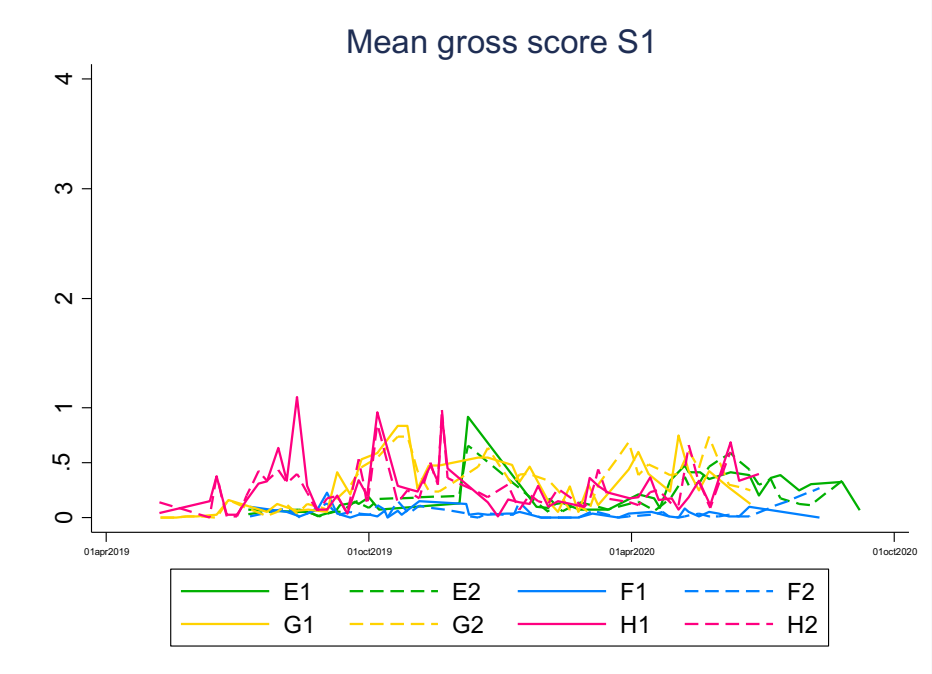


Figure 8. Mean gross gill score by stocking period (S0/S1) in farmed Atlantic salmon across eight marine sites in Norway. Top panel is autumn stocked fish, bottom is spring stocked. The scale ranges from 0-5.

4.2.3 Gill histology

Gills from the freshwater phase generally had minimal to mild pathology suggestive of overall good gill health, though samples were collected after the mortality attributed to salmon gill poxvirus disease (site D) had ceased. Mild epithelial cell necrosis or apoptosis was found in one or more fish in three project groups (A, D and H), while minimal to mild vascular lesions were found in gills from all six project groups and deformed gill filaments were found in 1 or more fish from five of the six project groups where samples were available.

During the sea phase of production median percent total histology scores were generally low with median scores below 2 percent across all time points and projects groups (Fig. 9). The exception to this was project groups G1 and G2, where an increase in median total histology scores to >8 % was observed in the first autumn and winter after sea transfer and coinciding with a high prevalence of *N. perurans* detected on qPCR and amoebae observed in the tissue.

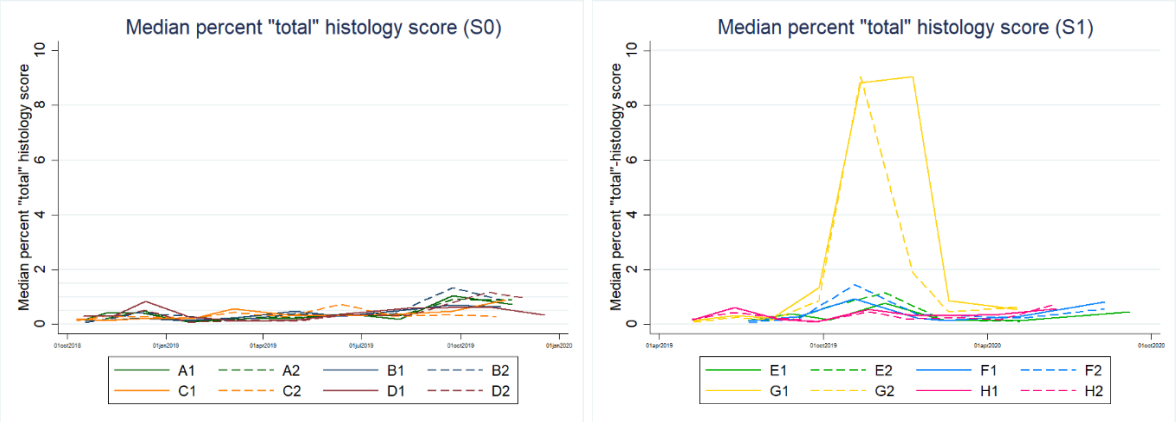


Figure 9. Median percent total histology score for stocking period (S0/S1) in farmed Atlantic salmon across eight marine sites in Norway. Left panel is autumn stocked fish, right is spring stocked. The scale range is from 0-100.

Median percent hyperplasia showed a similar pattern as percent total histology score across project groups and time points, and hyperplastic or hyperplastic together with inflammatory lesions contributed the most to the total score. The highest median percent hyperplasia was found in group G1 and G2, coinciding with the highest median total histology score. Median percent vascular lesions were generally less than 1 % for all project groups and time points, and the highest median percent vascular lesion were found in group G1 concurrently with the highest median percent total histology score. The median percent vascular lesions appeared to be increasing with time at sea (Fig. 10, 11 and 12), as illustrated below.

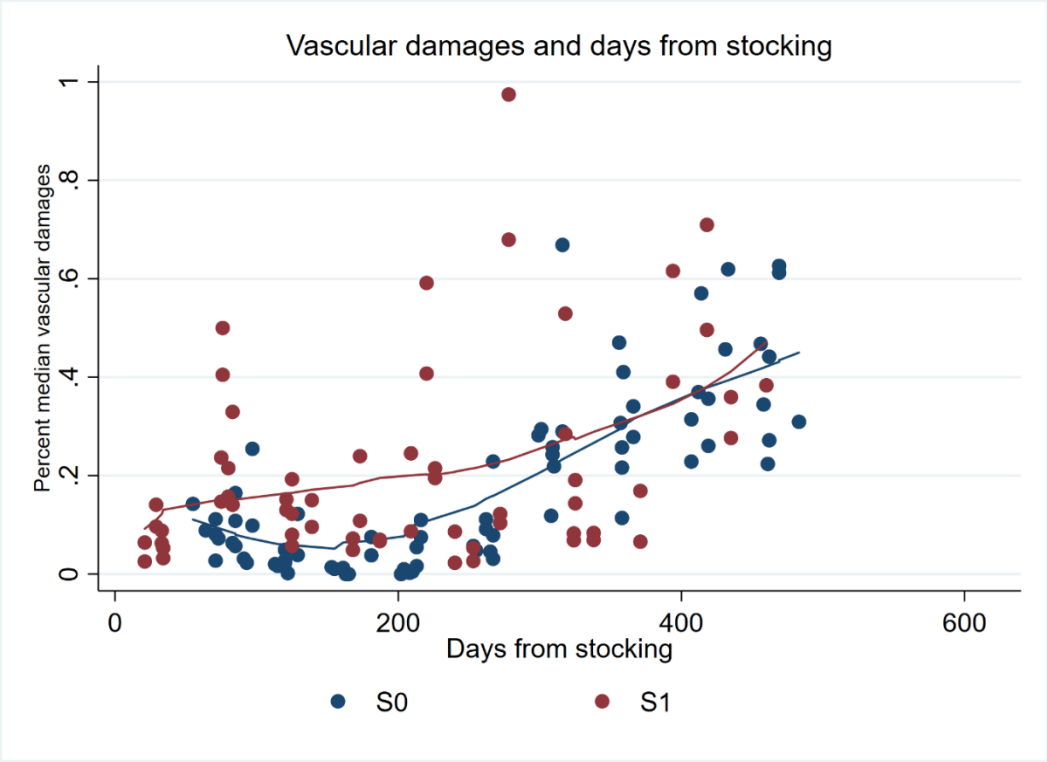


Figure 10. Percent vascular lesions in the gill tissue (group median) by days after stocking and stocking period (S0/S1) in farmed Atlantic salmon across eight marine sites in Norway.

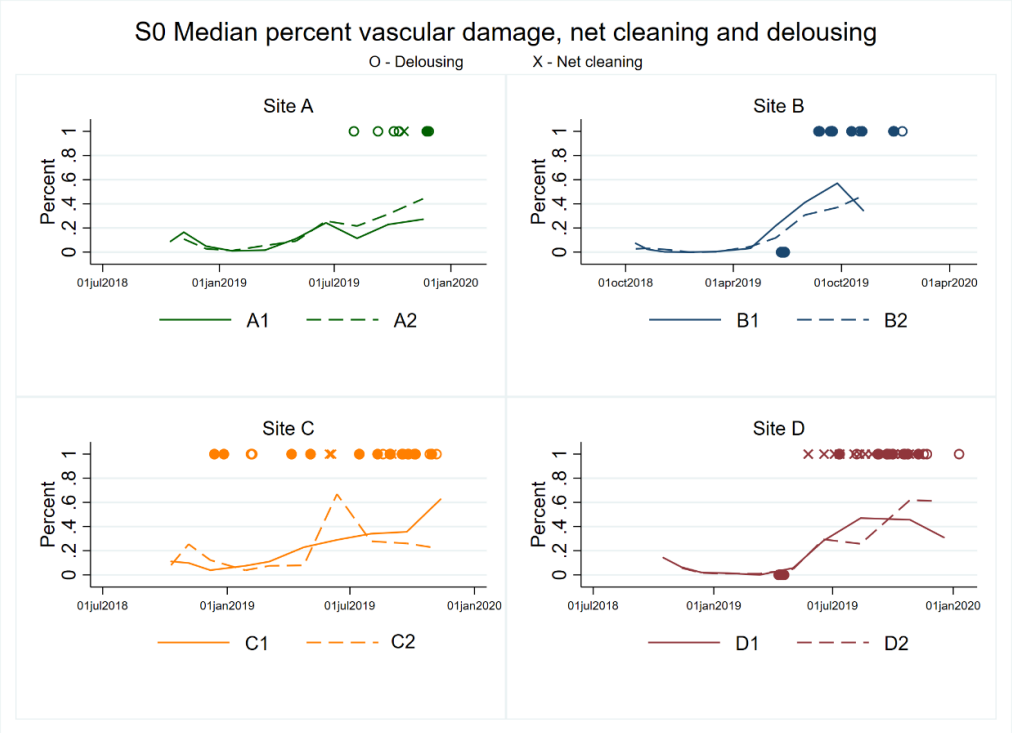


Figure 11. Median percent vascular lesions for S0 stocked Atlantic salmon by site, including days with net cleaning and delousing events.

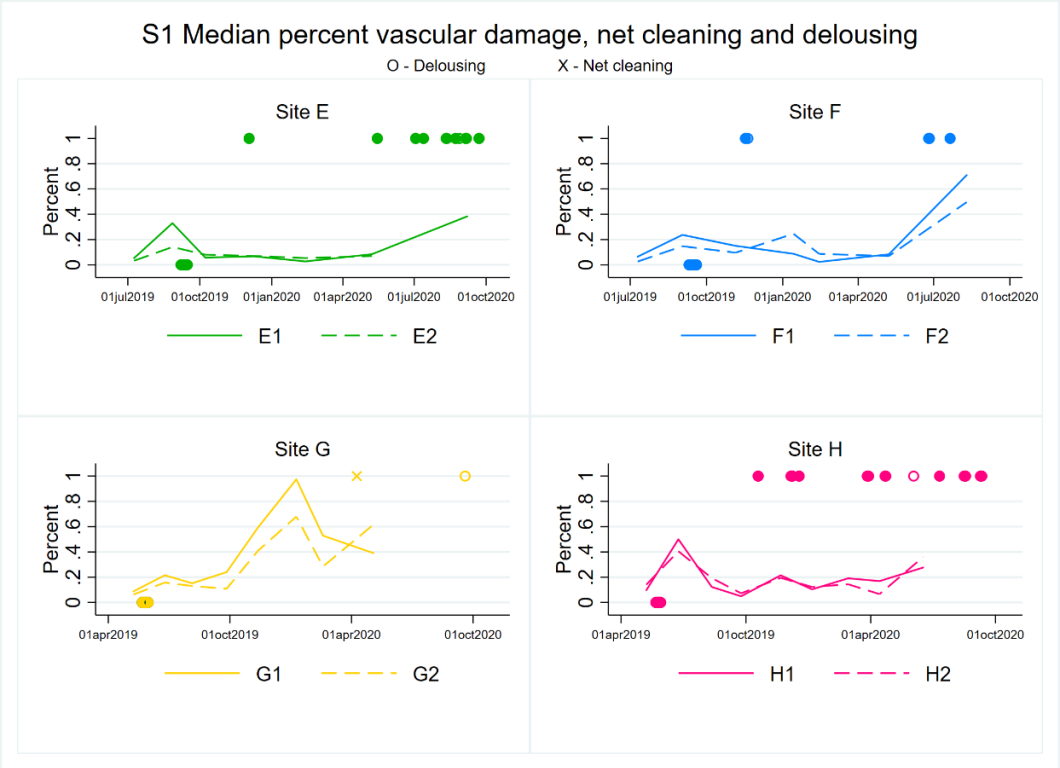


Figure 12. Median percent vascular lesions for S1 stocked Atlantic salmon by site, including days with net cleaning and delousing events.

Necrosis of lamellae was rarely observed in the material, and one or more samples with lamellar necrosis were found in 5 of 16 project groups during the sea phase, and in 1 of 8 fish groups in the freshwater phase. Necrotic lamella was most often associated with bacterial infection and/or foreign material trapped between filaments.

Amoebic gill disease as diagnosed by presence of amoeba and segmental lamellar epithelial hyperplasia (Fig. 13) was found in all but one project group (C2). This project group came from a site (site C) with considerable variation in salinity and at times brackish water. Similar to *N. perurans* detected by qPCR the diagnosis of AGD by histology showed a seasonal distribution and prevalence ranged from 0 to 100% across time points and project groups. A histology diagnosis of AGD was not necessarily coinciding with a marked increase in median percent total histology score, though the groups with the highest total histology score (site G) also had the highest prevalence of AGD diagnosed on histology. Free amoeba without associated gill histopathology were not identified in any of the samples.

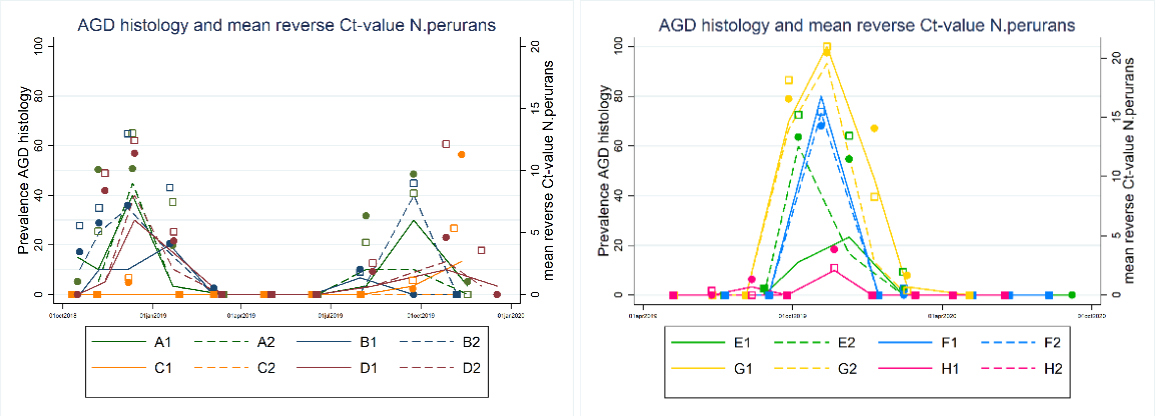


Figure 13. Prevalence of amoebic gill disease (AGD) diagnosed by histology and mean reverse Ct-values for *N. perurans.* Reverse Ct = 40 – Ct. Reverse Ct for negative samples were set to 0. Solid circles represent the Ct-values for 1 – groups, while hollow squares represent Ct-values for 2 – groups. Left panel is autumn stocked fish, right is spring stocked. The scale range is from 0-100.

Epitheliocysts were found in all project groups after sea transfer, but prevalence varied markedly between groups and time points. Epithelial cell necrosis and/or apoptosis was found at least on a sample at one or more time points in all fish groups, but prevalence varied from 0 to close to 80%. Filamentous bacteria, likely *Tenacibaculum* spp. (Fig. 4) were observed in just one fish and was associated with focal necrotizing branchitis. Intravascular bacteria and inflammation, haemorrhage and/or necrosis (Fig. 5) was found in 50% of fish during the last sampling of group C1 emphasizing that gill tissue can also be affected in cases of systemic bacterial infection. Other gill pathogens and parasites like *Trichodina* spp., *Ichthyobodo* spp. (Fig. 20), encysted metacercaria and small and large crustaceans were sporadically found within or associated with the gill tissue.

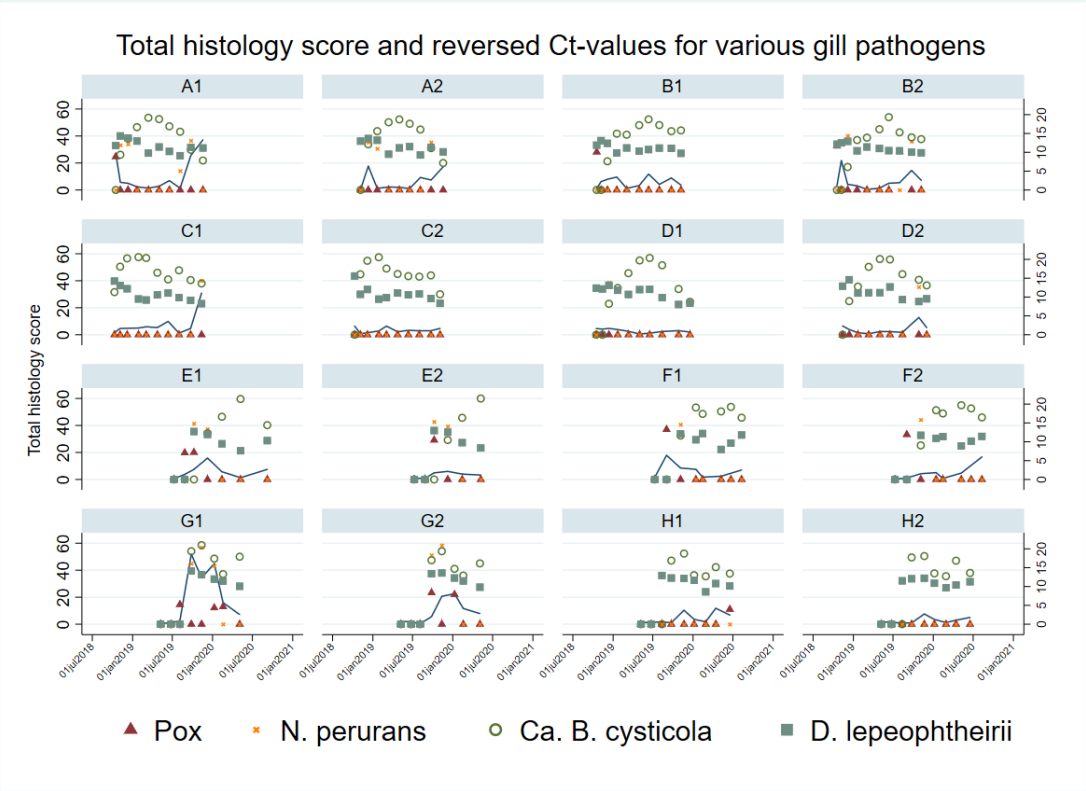


Figure 14. Maximum percent total histology score and median reversed Ct-values for selected pathogens per project group. Reverse Ct = 40 – Ct. Reverse Ct for negative samples were set to 0.

4.2.4 qPCR

During the freshwater phase SGP-virus was found in four fish groups (C, D, G and H), from two freshwater facilities (sites 3 and 4) and one group recorded SGPVD-related mortality (group D). *Ca.*B.cysticola was detected in one fish in group E, while the remaining samples and sites were negative. Presence of SGPV during the seawater phase seemed to have a seasonal distribution with positive samples collected in late summer and autumn in most sites (Figure 14). There was a variation in prevalence from 0-100% across groups, sites, and time points. However, all groups tested positive for SGPV at least once after sea transfer and mean Ct-values of the positive samples (per project group and time point) ranged from 26.9 to 36.8. After sea transfer all project groups became positive for *Ca.* B. cysticola and *D. lepeophtherii* and prevalence then remained high (60-100%) throughout the sea phase. Mean Ct-values of the positive samples (per project group and time point) ranged from 18.3 to 35.1 for *Ca.* B. cysticola and 25,2-32,3 for *D. lepeophtherii*. *N. perurans* was detected in all project groups but prevalence ranged from 0 to 100% positive samples across time points and project groups. The presence of the parasite showed a seasonal distribution, with positive samples late summer, autumn and winter and all negative samples in spring and early summer. Mean Ct-values of the positive samples (per project group and time point) ranged from 19.0 to 28.7

and the lowest mean Ct-values coincided with the highest prevalence of positive samples and with the highest prevalence of amoeba observed in tissue sections.

4.2.5 Production data, including overall - and gill related mortality

An overview of the cause-specific mortality, cumulative mortality, and results from qPCR analysis in freshwater is presented in Fig. 15. Mortalities are categorised by the site staff usually based on recommendation by fish health personnel. Fish health personnel is further involved should there be any suspicious or significant mortality events. Exactly what is considered a suspicious or significantly mortality may vary, but the Norwegian Food Safety Authority considers a daily mortality of 0,5 and 0,25‰ as significantly increased mortality for fish below and above 0,5 kg, respectively. Following the fish health persons visit and/or lab reports, the mortality categories can be retrospectively adjusted. When classification of mortality to one of the mortality categories is not possible, then the mortalities can be classified as 'unknown' or 'other'.

The accumulated mortality ranged from 4 to 8 percent across the included freshwater sites. One site (D) had recorded SGPV-related mortality.

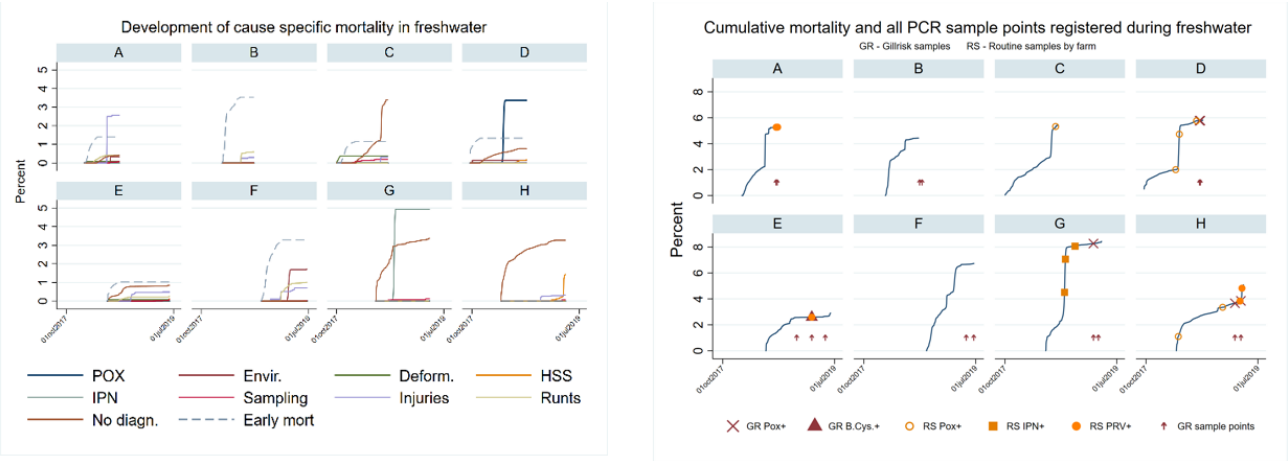


Figure 15. Cause specific mortality in freshwater (left) with cumulative mortality and PCR results in freshwater (right). GR indicate samples collected as part of the Gillrisk-project, while RS indicate routine diagnostic sampling.

Overall mortality in the seawater phase is presented in Figure 16, for S0 and S1 stocked salmon, respectively. An association was observed between delousing and accumulated mortality, as expected.

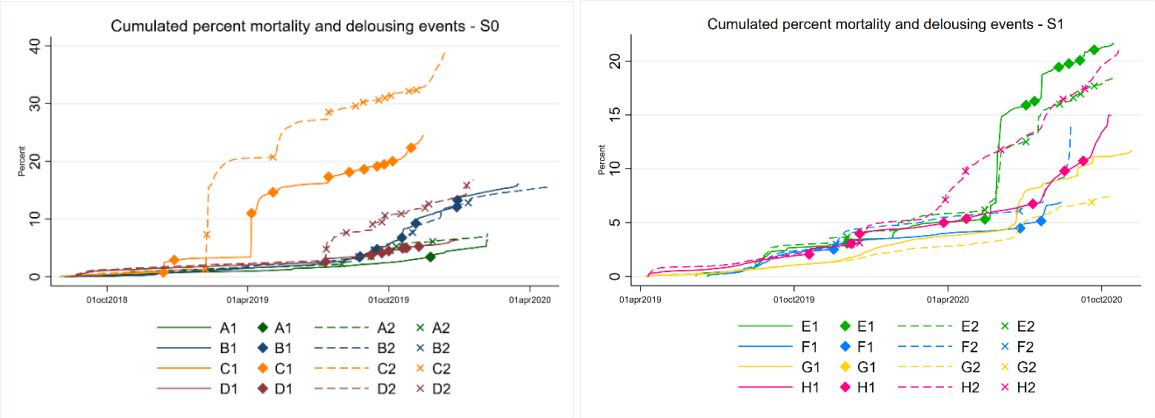


Figure 16. Percent mortality (accumulated) for S0 (left) and S1 (right) stocked Atlantic salmon by site and including days with delousing events (except in-feed treatments).

Relative causes of mortality in seawater are depicted in Figure 17. Delousing and handling are recorded as the leading cause of mortality in most sites, but infectious causes were also common followed by gill related mortality.

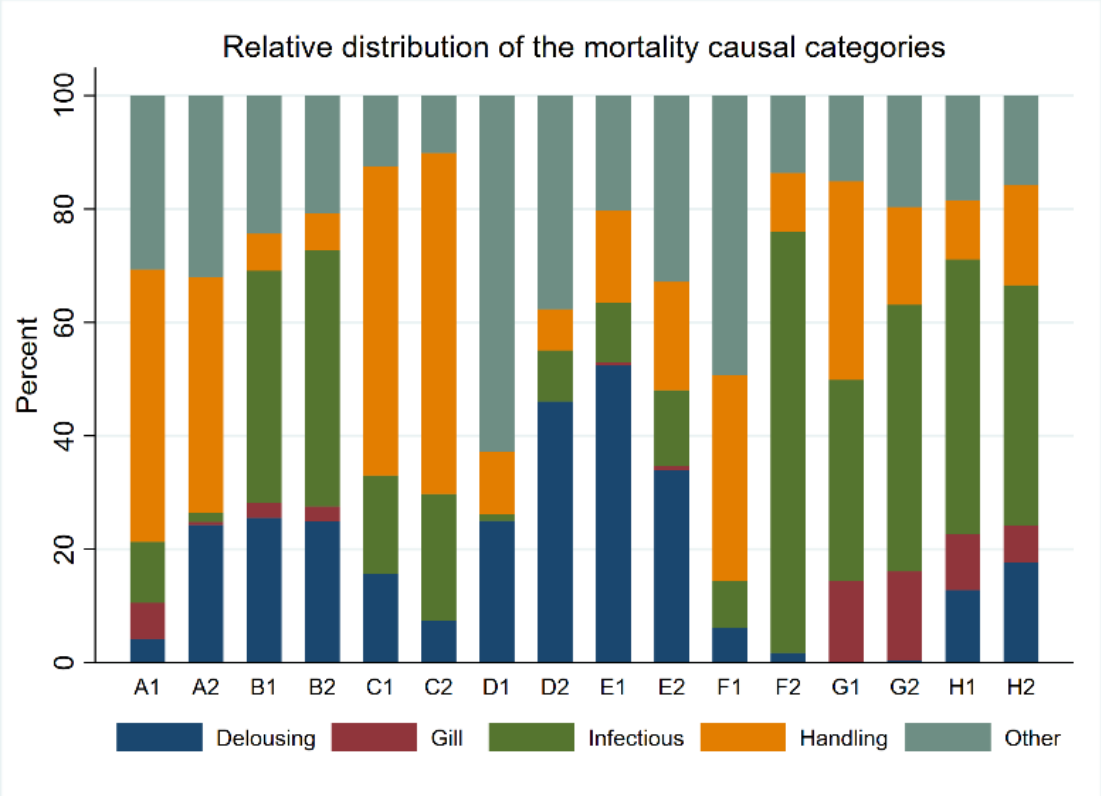


Figure 17. Relative distribution of causes of mortality across the marine sites from stocking to harvest.

As seen in Figure 18, gill related mortality increases during periods of higher water temperature. The association between gill-related mortality and Ct-values for the gill pathogens in qPCR-analysis are presented in Figure 19.

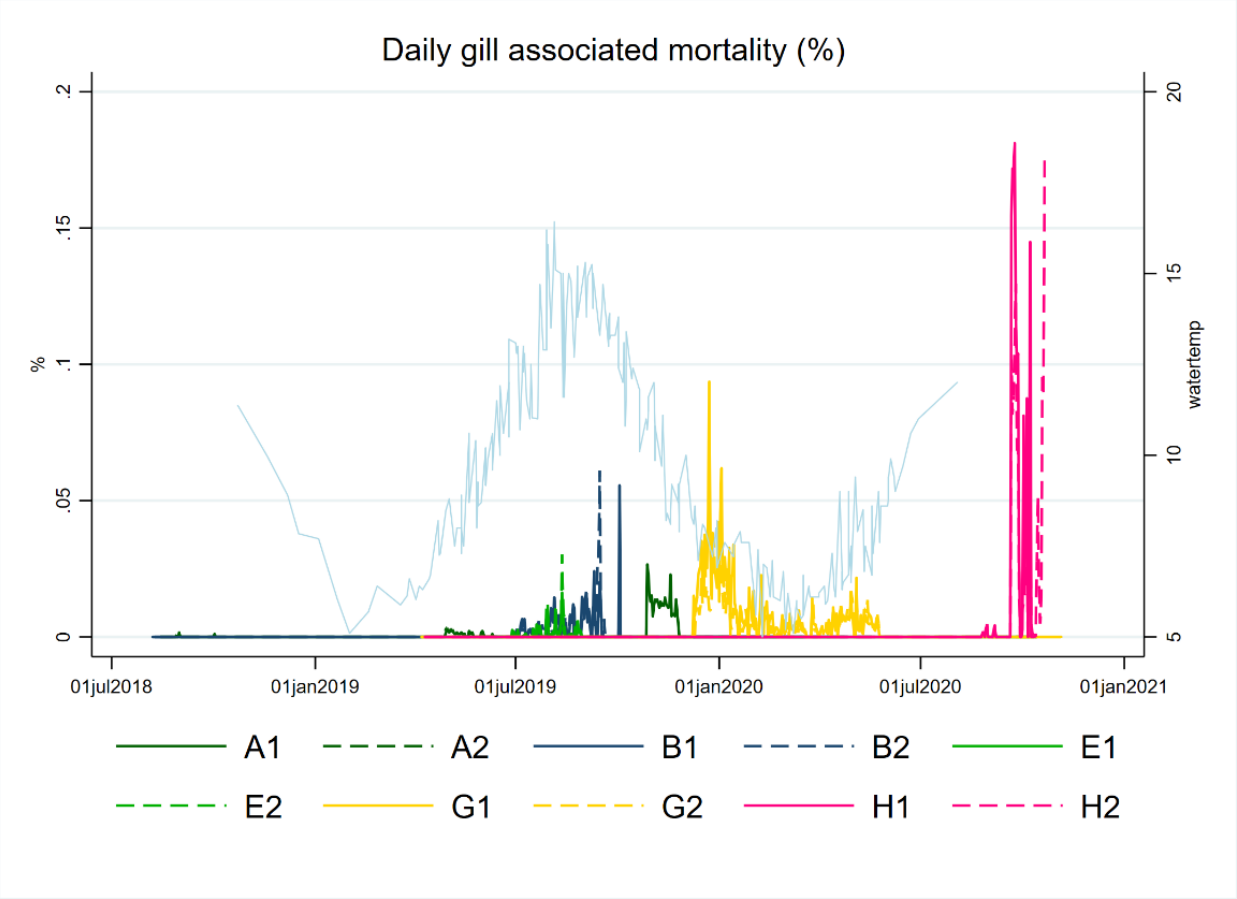


Figure 18. Daily percent gill-related mortality the per project group. The remaining 6 groups did not record any gill-related mortality.

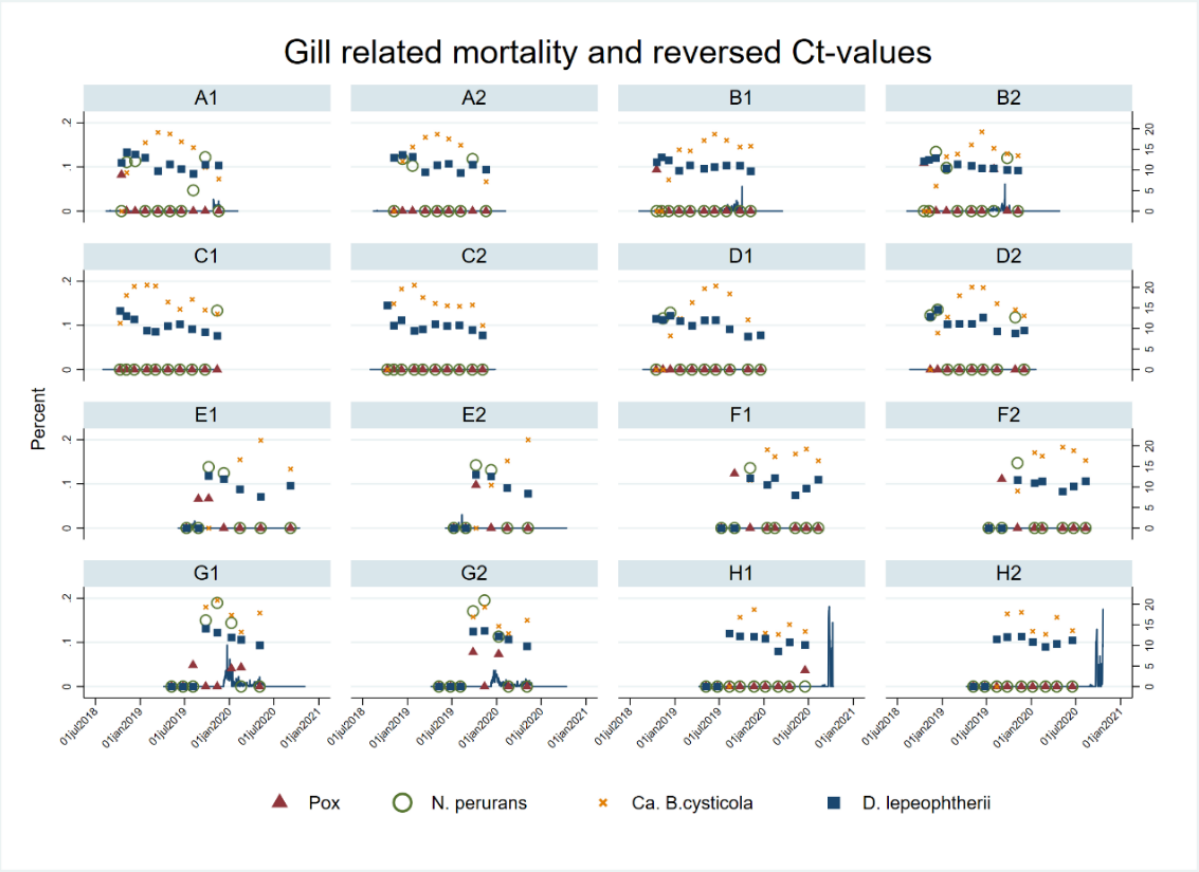


Figure 19. Daily percent gill-related mortality (blue line) by site and median reversed Ct-values for gill pathogens SGPV (pox), *N. perurans*, *Ca.* B. cysticola and *D. lepeophtherii*. Reverse Ct = 40 – Ct. Reverse Ct for negative samples were set to 0.

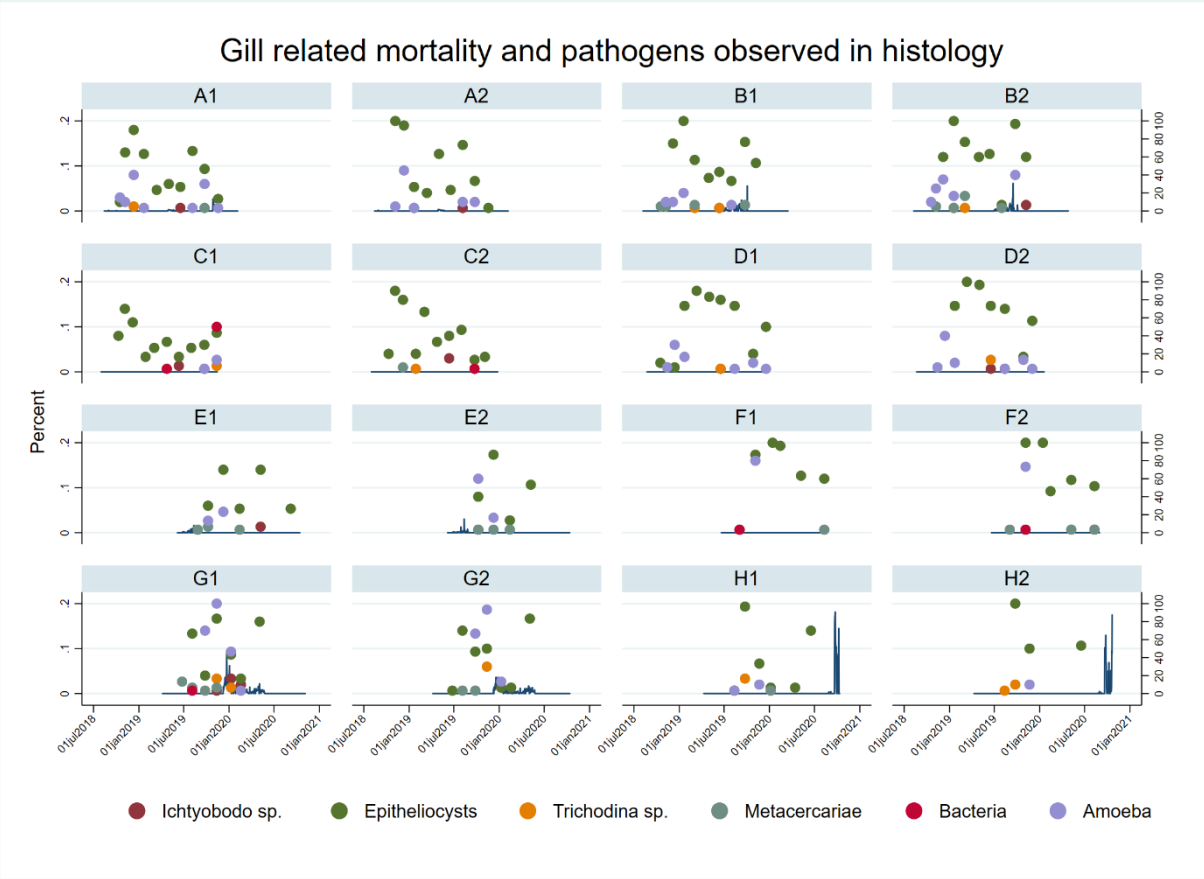


Figure 20. Pathogens observed on gill histology as well as recorded gill mortality in 16 groups of Atlantic salmon stocked at 8 marine sites in Norway during 2018 through 2020.

4.2.6 Factors associated with gill disease

Variable name	Stage	Origin of variable	Description
Amoeba	SW	Project samples	Histology samples where amoeba was observed, then aggregated as a percent on group level. The value has been repeated for 14 days.
Day degrees in fw	FW	Production data	Aggregated daily temperature in the freshwater production cycle
Delousing week	SW	Production data	Yes/no, non-feed delousing has taken place. Variable is kept as 1 for the week following a delousing operation.
Epitheliocysts	SW	Project samples	Histology samples where epitheliocysts was observed, then aggregated as a percent on group level. The value has been repeated for 14 days.
Gross score POL	SW	Collected by site staff as a part of project	Weekly gross score values, used a linear interpolation to get daily values by using “mipolate” in Stata.
Oxygen	SW	Production data	Sea oxygen levels registered by sea site staff
RAS	FW	Information from producer	RAS or flow-through freshwater site
Sea temperature	SW	Production data	Daily seawater oxygen levels
Weight spread coef.	FW	Weight spread coefficient before transfer to sea	The group weight spread factor calculated as the (SD weight/average weight) *100
Vascular lesions	SW	Project samples	Percent of gill tissue with vascular lesions detected on histology, aggregated as the median on group level. The value has been repeated for 14 days.

Table 3. Overview of the variables included in the models of association. The appendix contains a list of all variables tested in the univariable analysis.

4.2.6.1 Overall and gill related mortality during freshwater phase

The effect on total percent mortality in fresh water was investigated for several recorded factors such as O_2, pH and salinity, stocking period (S0/S1), vaccine used, number of moves and light regime. None of these factors were associated with increased mortality in the analysis. Water temperature was positively associated with log-transformed daily mortality in freshwater, while the association with CO_2 was negative as seen by the multivariable model in Table 4. Only one site experienced gill related mortality during the freshwater phase and no further analyses could be performed with gill related mortality as an outcome.

	Coef.	P	[95% Conf. Interval]	
Water temperature	0.1462	<0.001	0.1035	0.189
CO_2	-0.1132	<0.001	-0.1317	-0.0948
_cons	-5.642	<0.001	-6.201	-5.0831

Table 4. Linear mixed model of the effect of water temperature and CO2 concentration on log (percent daily mortality) in eight freshwater sites for Atlantic salmon.

The following variables from freshwater were selected for further investigation regarding possible associations with gill disease during the sea phase:

RAS/FT
Stocking season (S0/S1)
Number of moves
Light regime
Number of days with high CO_2 (above 15mg/l) in FW
Total mortality in fresh water
Vaccine type
Day degrees
Weight distribution before transfer

4.2.6.2 Overall mortality and gill related mortality during sea phase.

Overall mortality

The effects of freshwater factors on overall mortality were investigated using linear regression, after initial screening by univariable analyses as described above. Three separate univariable analyses were performed, and results are presented in Appendix 4. Factors found to be significantly associated with the outcome variables (liberal cut-off for inclusion at p<0.2) were offered to the three models for the first 90 days in sea (Table 4), the first 180 days (Table 5) and the full seawater phase (Table 6), respectively. The outcome was log-transformed percent daily mortality in all models.

First 90 days after stocking

When controlling for group and site, there was a significant association between “day degrees in freshwater” and “log (percent daily mortality)” for the first 90 days after stocking of 0.0012 (p=0.003). The coefficient for seawater temperature was 0.1397 (p<0.1073). By using the inverse logarithm, the interpretation of the coefficients is that for each one-unit increase in “day degrees” the percent daily mortality increases by 1.00 and for a one-unit increase in seawater temperature the mortality increases by 1.39. This corresponds to an increase in percent daily mortality of 2.28 across the interquartile range (IQR) of “day degrees” (2728 to 3096) for the first 90 days after stocking. For “seawater temperature”, the increase is 2.9 percent daily mortality (IQR: 10.4C to 13.53C).

	Coef.	P	95% conf. Interval	
Day degrees in freshwater	0.0012	0.003	0.0004	0.0021
Weight spread at stocking	-0.0518	0.075	-0.1088	0.0052
Seawater temperature	0.1397	<0.001	0.1073	0.1722
_cons	-9.4520	<0.001	-11.8243	-7.0798

Table 5. Linear mixed model “log transformed total mortality first 90 days after stocking”.

First 180 days after stocking

There were no significant associations between recorded freshwater factors and percent daily mortality during the first 180 days after stocking. When controlling for group and site, there was a significant association between “seawater temperature” and “log (percent daily mortality)” during the first 180 days after stocking with a coefficient of 0.1498 ($p<0.001$). The coefficient for “gross score” was 0.5612 ($p<0.001$). By using the inverse logarithm of the coefficients, the interpretation of is that for each one-unit increase in “seawater temperature” the percent daily mortality increases by 0.15 and for a one-unit increase in seawater temperature the mortality increases by 0.56. This corresponds to an increase in daily mortality of 5.42 percent across the interquartile range (IQR) of “seawater temperature” (8.5C to 13.4C) for the first 180 days after stocking. For “gross score”, the increase is of 1.51 on the observed percent daily mortality (IQR: 0.073 to 0.393).

	Coef.	P	[95% Conf.Interval]	
Seawater temperature	0.1498	<0.001	0.1287	0.1708
Gross score	0.5612	<0.001	0.3006	0.8218
_cons	-6.7558	<0.001	-7.0937	-6.4179

Table 6. Linear mixed model “log transformed total mortality first 180 days after stocking”.

Full seawater cycle

No association between the recorded freshwater factors and overall mortality for the full stocking period was observed. When controlling for group and site, there was a significant association between “seawater temperature” and “log (percent daily mortality)” for the full stocking period with a coefficient of 0.0624 ($p<0.001$). The coefficient for “gross score” was 0.9089 ($p<0.001$) and for “delousing week” it was 1.0559 ($p<0.001$). By using the inverse logarithm of the coefficients, the interpretation is that for each one-unit increase in “seawater temperature” the percent daily mortality increases by 1.15, for a one-unit increase in "gross score” the mortality increases by 8.11. This corresponds to an increase in daily mortality of 2.16 percent across the interquartile range (IQR) of “seawater temperature” (7.4˚C to 12.75˚C)

for the full stocking period. For “gross score”, the increase is of 2.05 on the observed percent daily mortality (IQR: 0.1159 to 0.4586). The coefficient for delousing week is exponentiated to 11.38 which means that in the week following a delousing episode the mortality was 11.4 percent higher than in weeks with no delousing (delousing here is not including in-feed treatments).

	Coef.	P	[95% Conf. Interval]	
Sea temperature	0.0624	<0.001	0.0529	0.0718
Gross score	0.9089	<0.001	0.8193	0.9985
Delousing	1.0559	<0.001	0.9555	1.1563
_cons	-5.6145	<0.001	-5.8547	-5.3743

Table 7. Linear mixed model “log transformed total mortality, full seawater cycle”.

Gill related mortality

The effects of factors in fresh- and seawater on gill related mortality were investigated using initial univariable negative binomial regression, controlling for fish-group and site. Results (Table 8) indicate that median percent vascular lesions are associated with gill related mortality. The interpretation of the coefficient for vascular lesions is that for every one-unit increase in percent of vascular lesions in the gill tissue the expected log count of number of dead fish increases by 0.43. This corresponds to 8 dead fish across the IQR of percent vascular lesions (0.5979 to 2.6842).

	Coef.	P	[95% Conf.	Interval]
Vascular lesions	0.4326	0.019	0.0701	0.7950
_cons	-6.0608	<0.001	-9.0897	-3.0319

Table 8. Negative binomial mixed model “gill associated mortality, full seawater cycle”.

Gill histology

RAS, gross score, seawater oxygen and amoeba detected by histology examination came out as being significantly associated with total gill histology score in this analysis. Coefficients and p-values are listed in Table 9 below. The interpretation of the coefficient for RAS is that fish groups from RAS sites had 0.34 percent worse total histology results than fish groups from flow-through sites, while fish groups where amoeba was detected had 0.019 percent worse histology. All in all, the results indicate that these factors had a very small effect on the total histology results on group level. The model further estimates an increase in total histology results of 0.40 percent across the IQR of gross scores (0.1159 to 0.4586) and a decrease of -0.20 percent across the IQR of oxygen levels in seawater. The negative coefficient for oxygen levels shows that lower oxygen levels are associated with higher histology scores. Values for

histology scores were “carried through” or repeated daily for 14 days after tissue samples were collected.

	Coef.	P	[95% Conf.Interval]	
RAS	0.3435	0.012	0.0768	0.6102
Gross score	1.1635	<0.001	0.9920	1.3351
Oxygen	-0.0183	<0.001	-0.0249	-0.0116
Prevalence amoeba histology	0.0199	<0.001	0.0163	0.0236
_cons	2.3029	<0.001	1.6178	2.9881

Table 9. Linear mixed model for total histology score.

6. WP2 – Evaluation of serum biochemical and blood gas parameters as potential indicators of gill function and evaluation of usefulness of point-of-care blood analysis as an on-site diagnostic tool

Due to concerns about the reliability of the selected point of care device and the usefulness for blood analysis for assessment of gill function two small scale pilot studies and a brief literature review was performed.

1. To test the concordance between a point of care (POC) device (VetScan iSTAT1 handheld blood analyzer) and a laboratory blood analysis machine (ABX Pentra C400), blood samples from 21 Atlantic salmon post-smolts (200 g) at the NIVA Research Facility at Solbergstrand were analyzed. Blood samples were analyzed for sodium (Na), potassium (K), chloride (Cl), haematocrit (Hct) and lactate.

2. To examine possible co-variation in gross and histological gill scores and selected blood parameters and get more data on to assess concordance between the laboratory and POC device, 41 Atlantic salmon (2.4-4.9 kg) from a sea site in Møre og Romsdal with grossly visible gill lesions and a history of recent gill disease, were sampled.

Sampling study 1

The 21 fish were euthanized with an overdose of benzocaine and blood was collected from the caudal vein into lithium-heparinized tubes. iSTAT analysis was performed on whole blood as soon as possible after sampling, however, time from sampling to analysis varied from a few minutes to approximately 1.5 hours. The delay between sampling and analysis is likely to have led to an increased temperature of the affected samples. The remaining heparinized blood was cooled, centrifuged and plasma was transferred into 2 ml Eppendorf tubes and chilled until arrival at the lab and subsequently frozen until analysis at the lab.

Sampling study 2

The 41 fish were sedated with benzocaine as per producer recommendations for lice counting. After lice counting, the fish were placed in a saltwater tank and sedated with isoeugenol and subsequently euthanized with an overdose of metacaine. The fish were euthanized and sampled one at a time. Immediately after euthanasia, blood was sampled as described for Study 1, gross gill scoring was performed as described in section 4.2.2.and the second gill arch on the left side and the heart sampled for histology. iSTAT analysis was performed on each sample within 5 minutes after sampling, while plasma for Pentra C400 analysis was stored at -20°C and shipped on ice to Fish Vet Group lab.

Pentra C400 analyses for both sample sets were performed at the Fish Vet group lab at Skøyen, Oslo. Plasma samples were analyzed for Na, K, Cl, and lactate using ABX Pentra kits according to instrument protocols.

Histological gill score was based on the distribution and severity of lamellar inflammation, lamellar epithelial hyperplasia, and vascular damage. Zero represented no lesions, 1 mild and focal lesions, 2 moderate and multifocal lesions and 3 severe and multifocal to coalescing lesions. No cardiac lesions were observed in 28 fish, while minimal, non-specific lesions (inflammation, haemorrhage, or thrombi) were found in the remaining 13 fish.

Statistical analysis of results from both studies were done in STATA. Direct agreement (concordance) between the laboratory-based machine and the POC-device was assessed by the concordance correlation coefficient. Correlation between the two readings was also assessed by Pearson's correlation coefficient. Co-variation between biochemical parameters and gross and mean histological scores were assessed visually using Box-and-whisker plots.

Results

Practical experience with the iSTAT and agreement between iSTAT and Pentra C400

The iSTAT is portable and easy to use, however the device requires a stable temperature of 16-30°C to function optimally and both the device and the cartridges need to be acclimatized to the temperature in the room used for analysis. In our experience the iSTAT should acclimatize for at least 1 hour on site prior to analysis. The device should not be placed near sunny window or other heat sources. In addition, it requires a stable surface. These requirements make the use of the device less practical for on-site use at fish farms. The device and the cartridges are easy and intuitive to use. The time for each separate cartridge to perform the analysis in Study 1 was approximately 8 minutes. In total analysis of each sample (2 cartridges per sample) took approximately 20 minutes from start to finish. In Study 2, each separate cartridge took approximately 4 minutes, and each fish took approximately 10 minutes to sample in total.

Results of blood and plasma analysis are summarized in Table 10. The iSTAT gave no valid readings from a relatively high proportion of the samples, 19/62 (31%) and 10/62 (16%) for K

and Cl, respectively. Additionally, a large fraction of readings was below the highest or lowest detectable levels for both ions, leaving only 10 duplicate measurements for Cl and 33 duplicates for K. In contrast all samples analyzed with the Pentra resulted in presumed valid readings for all analytes. The iSTAT lactate measurements ranged from 0.69 to 2.42 mmol/l. Plasma lactate measured by the Pentra ranged from 1.03 to 3.47 mmol/l. Converted lactate values were generated by multiplying plasma lactate values by 1– haematocrit.

	Na mmol/l (Lab)	**Na mmol/l (POC)**	**K mmol/l (Lab)**	**K mmol/l (POC)**	**Cl mmol/l (Lab)**	**Cl mmol/l (POC)**	**Lactate mmol/l (Lab)**	**Lactate mmol/l (Lab con†)**	**Lactate mmol/l (POC)**
Study 1	155.7 (17.8) n = 20	156.5 (8.78) n = 21	1.78 (1.29) n = 20	2.0 (0.4) n = 3	138.8 (16.6) n = 20	130.7 (6.1) n = 11	2.36 (0.76) n = 20	1.66 (0.50) n = 20	1.57 (0.51) n =21
Study 2	167.7 (11.5) n = 41	166.4 (6.0) n = 31	1.70 (1.25) n = 41	3.2 (0.8) n = 31	141.6 (9.6) n = 41	- n = 0	12.25 (6.0) n = 41	ND	ND

Table 10. Mean values for analytes as measured by the POC (iSTAT) and laboratory analyser (Pentra C400). Standard deviation in parentheses. Mean values are calculated for numerical values only. Calculation of mean values was not possible for the analyte Cl for one or both samplings due to no and few samples with numerical values. ND = not done. †Converted values generated by multiplying original values with a conversion factor C (C = 1– haematocrit/PCV).

Both the correlation and concordance correlation coefficient (Table 11) and scatter plot indicated poor agreement between the two devices for chloride, sodium, and potassium (data not shown). The scatterplots (Fig. 21 and 22) and Pearson correlation coefficient shows a high correlation between devices for both converted and unconverted lactate measurements, while concordance is high only between iSTAT and converted Pentra lactate values. Results of the agreement analysis have been published and this manuscript is available as Appendix 5).

	Na mmol/l	**K mmol/l**	**Cl mmol/l**	**Lactate mmol/l**	**Lactate converted mmol/l**
Study 1	0.16 (0.23) n =20	-	- 0.43 (- 0.52) n= 10	0.49 (0.95) n = 20	0.89 (0.91) n = 20
Study 2	0.43 (0.47) n = 31	0.09 (0.30) n = 30	-	-	-
Overall samples	0.38 (0.44) n = 51	-0.03 (-0.07) n = 33	- 0.52 (- 0.43) n = 10	0.49 (0.95) n = 20	0.89 (0.91) n = 20

Table 11. Agreement between the POC-device and laboratory analyser. Concordance correlation coefficient and Pearson's correlation coefficient (in parentheses), n = number of observations.

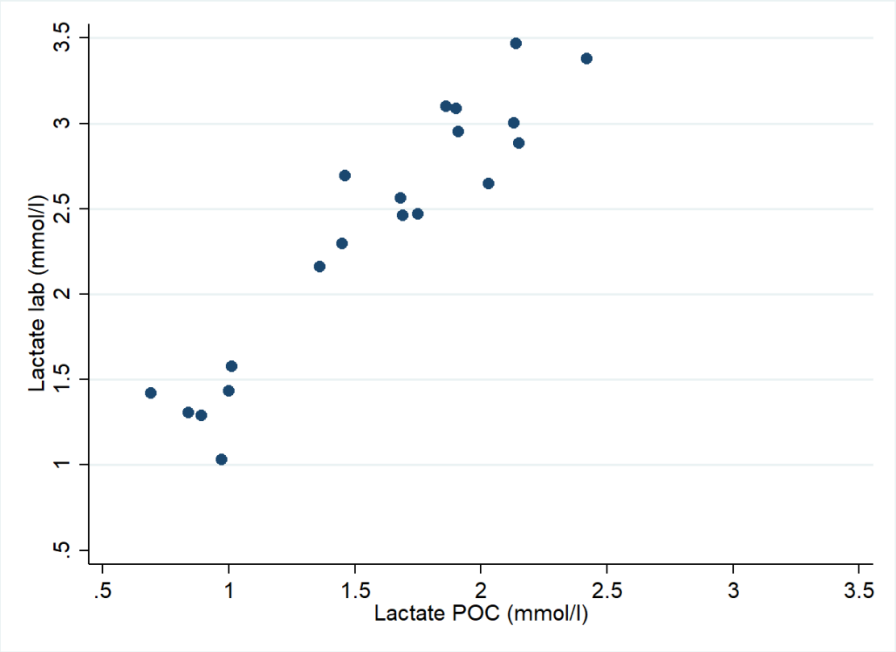


Figure 21. Scatterplot of lactate levels as measured by iSTAT and Pentra. (Data from Study 1).

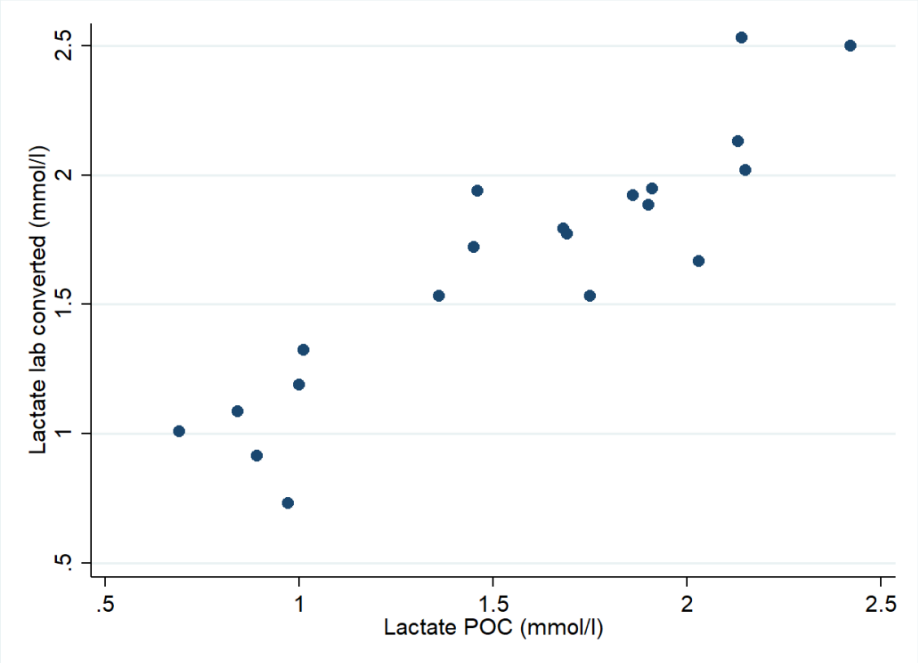


Figure 22. Scatterplot of lactate levels as measured by iSTAT and converted lactate levels measured by Pentra. (Data from Study 1).

Co-variation in gross and histological gill scores and selected blood parameters.

Gross scores were summarized as a mean gross gill score for each fish and the mean scores were used for further analysis. Most sampled fish received low gross and histological gill scores (Table 12 and 13). None of the examined analytes (sodium, potassium, lactate, or total CO_2) seemed to be positively or negatively associated with changes in gill health status as measured by gross and histological gill scores (Fig. 23 and 24). However, as Table 12 shows, most fish (31/41 fish) have mean gross gill scores equal to or less than 1. Similarly, 38 fish were given a mean histologic gill score of 1 or less. Only 3 fish had a mean histologic gill score of 2 and no fish reached a histologic score of 3, 4 or 5. These low scores indicates that most of the gill tissue was healthy and even if some functionality might have been lost, the lack of correlation between gill health status and blood parameters in our study could be due to the relative mild gill lesions.

Mean gross gill score	*Number of samples per score*
0	7
0.25	5
0.5	11
0.75	4
1	4
1.25	4
1.75	3
2	2
3	0
4	1
5	0

Table 12. Distribution of gross gill scores.

Histological gill score	*Number of samples per score*
0	18
0.5	5
1	16
2	3
3	0
4	0
5	0

Table 13. Distribution of histological gill scores.

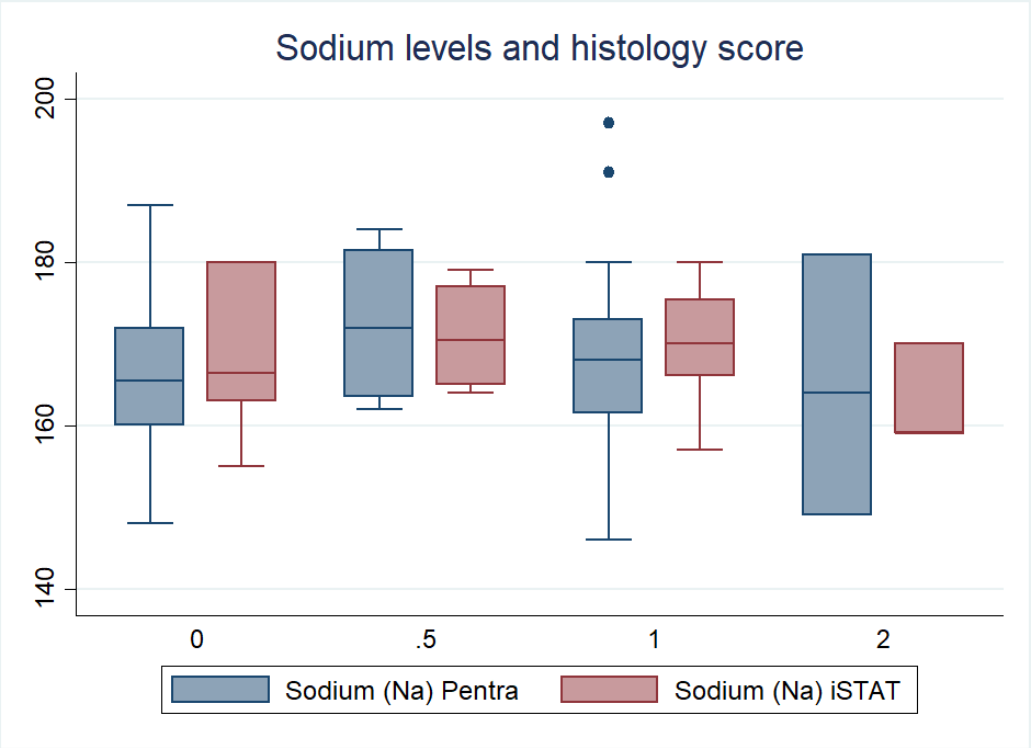


Figure 23. Boxplot of histological gill scores (x-axis) plotted against blood and plasma sodium levels (y-axis).

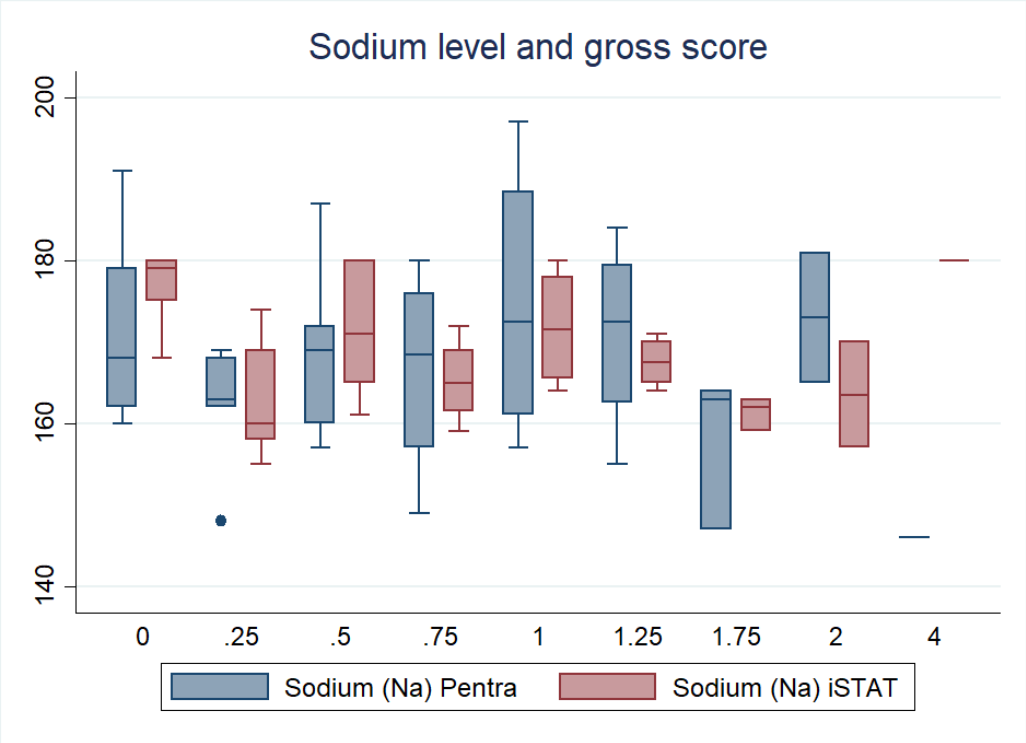


Figure 24. Boxplot of gross gill scores (x-axis) plotted against blood and plasma potassium levels (y-axis).

Literature review

Blood gas as an on-site indicator of gill health?

Blood gas levels have been shown to be affected by gill pathology in Atlantic salmon experimentally infected with *Neoparamoeba* sp. (Powell, et al., 2000). Collection of arterial blood in fish without euthanasia requires previous surgical cannulation of the dorsal aorta. Blood gas levels in both venous and arterial blood will be affected by anaesthesia as most anaesthetics intended for sedation and anaesthesia of fish induces respiratory depression. However, blood sampling of unanaesthetized, non-cannulated and living fish cannot be done without compromising fish welfare. Additionally, the blood gas levels will rapidly change after sample collection, so storage and later analysis is not possible. Although we did not test the reliability of the iSTAT system for blood gas analysis, previous work suggests that iSTAT measurements are not useful for PCO_2, PO_2, bicarbonate or sO_2 in fish. Taking all these factors together blood gas analysis using iSTAT is not practical for evaluation gill function in a clinical setting.

Lactate and ions as indicators of gill health status

The gills and chloride cells excrete excess sodium and chloride during the marine phase, and it is possible that gill disease could interfere with this function. Hvas et. al (Hvas, et al., 2017) found elevated plasma levels of chloride, sodium, potassium, cortisol, and lactate in salmon with AGD compared to non-infected salmon and attributed the elevated ion and cortisol levels to chronic stress and problems with maintaining homeostasis. Elevated ion levels and plasma lactate was also suggested to result from the presumed reduced functional gill area due to severe gill hyperplasia. On the other hand, Powell et al. (Powell, et al., 2004) found no significant differences in plasma chloride levels between Atlantic salmon experimentally infected with *Tenacibaculum maritimum* and non-infected control fish. A subset of moribund infected fish had significantly elevated lactate levels, which the authors attributed to possible acute respiratory failure. However, lactate levels can increase in a range of conditions causing local or systemic hypoxia and/or hypoperfusion such as during excessive muscle activity and are not gill-specific. Increased lactate levels were found in salmon with infectious salmon anaemia and myopathies such as in pancreas disease (PD). Lactate is used as an indicator of stress during handling and lactate levels is also affected by time in anaesthetic baths. Thus, there are multiple causes of elevated lactate levels and standardization of handling and anaesthesia time is necessary for sensible interpretation of this parameter.

7. WP3 – Acute effects of thermal or mechanical delousing and net cleaning on gill health of Atlantic salmon

6.1 Material and methods

6.1.1 Fish, sites, sampling, and exposures

Thermal delousing

The sea site selected for thermal lice treatment was in Vestland county in Western Norway and the fish were sea transferred in November 2018. The fish had not been treated for salmon lice previously and there were no known disease problems at the site. Mean fish weight, fish number and biomass per pen on the treatment day is summarized in Table 14. The month before treatment sea temperatures ranged from 12.8 to 15.7°C and oxygen saturation ranged from 44 to 87%. Treatment of the fish enrolled in this study was performed in September 2019 using a well-boat, with two treatment lines each with one Thermolicer unit. Crowding time for study pens ranged from 51 to 60 minutes, and time from start to end of treatment ranged from 2 hours and 15 minutes to 3 hours per pen. The treatment water temperature was 33.9°C and time in treatment loop was 28 seconds, while the sea temperature was 15.5°C. No issues were reported during the treatment.

Mechanical delousing

The sea site selected for mechanical delousing was in Trøndelag county in Northwestern Norway and fish were sea transferred in September 2019. The fish had not been treated for salmon lice previously and there were no known gill health issues at the site. Heart and skeletal muscle inflammation (HSMI) were confirmed in pen B 14 days prior to delousing and also in two other pens not included in the study. The month before treatment sea temperatures ranged from 8.53 to11.91°C, 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 14. The 3 study pens were deloused July 2020 and treatment was performed using a vessel containing three FLS delousing lines delivered by Flatsetsund Engineering in 2017. Crowding time prior to treat was approximately 1 hour and total time used from start of crowding to end of treatment ranged from 4.2 to 6.28 hours. Flushing pressure of the water jets was 0.76 to 0.77 BAR for the pens included in this study. According to the manufacturer the estimated treatment time per fish is 2 seconds, while time through the entire system is 10 to15 seconds per fish.

Net cleaning

The study site was a commercial sea site in Møre og Romsdal county in Northwestern Norway and consisted of six pens with Atlantic salmon. The fish had not been deloused and the nets

had not been washed since the fish arrived at the site. There were no known gill health issues, issues related to plankton or jellyfish or other diseases at the site. The month before net washing sea temperatures ranged from 9.5 to 12.1 °C and oxygen saturation ranged from 80.3 to 96.3 %. Salinity was >30 ‰ and there were no significant fluctuations in salinity level at the site. Fish were kept in multifilament nylon nets with small to medium mesh-size coated with Netwax GreenlineE5 (Netkem) which contains 25-30% copper oxide (Cu_2O and CuO). Net washing and sampling were performed in late November 2020. The level of net fouling prior to net cleaning was scored based on photos using a semiquantitative scoring system ranging from 0 to 6, i.e., from totally clean to heavy fouling. The net fouling level was low in pen C and moderate in pens A and B. All three project pens were washed on the same day using a Manta net cleaner (Stranda Prolog).

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	A	3,5	42 289	149 135
	B	3,0	98 520	294 692
	C	3,1	64 871	199 689
Net cleaning	A	0,73	158 810	116 127
	B	0,70	161 189	113 536
	C	0,55	184 355	99 128

Table 12. Mean fish weight, fish count and biomass for each pen at the day of net cleaning or delousing.

Sampling

Thirty fish per pen were sampled prior to treatment, at least 24 hours after treatment and at 6 to 9 days after treatments. 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 when sampling after thermal delousing or net cleaning. No fish with clinical signs of disease or moribund fish were observed or sampled after mechanical delousing either, except for two moribund fish sampled from pen C at six days after treatment.

Laboratory analysis

Gill tissue was processed and read as described in section 4.1.5 with the following exceptions: Slides were examined and scored in a random order and the pathologist was “blinded” regarding pen and time point of sampling relative to delousing or net cleaning and results of qPCR-analysis if available. Inflammation of filaments was recorded as a dichotomous variable and haemorrhage was counted and included in the lesion category vascular lesions. The same

pathologist read all samples from each of the three exposures, but one pathologist read delousing samples (LØ) and one read net cleaning samples (HR). A subset of samples collected during thermal delousing was examined with RT-qPCR for gill pathogens *Candidatus* Branchiomonas cysticola*, Paranucleospora theridion/Desmozoon lepeophtherii*, salmon gill poxvirus (SGPV) and *Neoparamoeba perurans* (table 15). Normalized Ct-values were calculated for pathogens *Ca.* B. cysticola and *D. lepeophtherii* using the following formula:

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

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

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

Pen	*Sampling 1*	*Sampling 2*	*Sampling 3*
A	Histology n=30 qPCR (*D. lep* & *Ca.* B. cys) n=18 qPCR (*N. per* & SGPV) n=5	Histology n=29 qPCR (*D. lep* & *Ca.* B. cys) n=18 qPCR (*N. per* & SGPV) n=5	Histology n=30 qPCR (*D. lep* & *Ca.* B. cys) n=30 qPCR (*N. per* & SGPV) n=30
B	Histology n=30 qPCR (*D. lep* & *Ca.* B. cys) =18 qPCR (*N. per* & SGPV) n=5	Histology n=30 qPCR (*D. lep* & *Ca.* B. cys) n=18 qPCR (*N. per* & SGPV) n=5	Histology n=30 qPCR (*D. lep* & *Ca.* B. cys) n=30 qPCR (*N. per* & SGPV) n=25
C	Histology n=30 qPCR (*D. lep* & *Ca.* B. cys) n=18 qPCR (*N. per* & SGPV) n=5	Histology n=30 qPCR (*D. lep* & *Ca.* B. cys) n=18 qPCR (*N. per* & SGPV) n=5	Histology n=30 qPCR (*D. lep* & *Ca.* B. cys)) n=30 qPCR (*N. per* & SGPV) n=25

Table 13. Overview of samples and analysis performed for the thermal delousing study.

Statistical analysis

Statistical analysis of results was undertaken in STATA. Box and whisker plots were produced to provide a visual presentation of the percent gill tissue affected by hyperplasia and vascular

lesions and fold change of normalized *Ca.* B. cysticola and *Desmozoon lepeophtherii* per pen and time point. To determine if there was as significant change in the number of gill lesions before and after delousing, we used a series of negative binominal regression models, while logistic regression models were used for dichotomous variables (number of fish with a given lesion). To assess whether there was a significant increase in genetic material of pathogen *Ca.* B. cysticola (Fold change of normalized Ct-values) we used a linear regression model. A Kruskal-Wallis test was used to assess possible differences between time points for fold change of normalized *D. lepeophtherii* Ct-values as criteria for normality were not met. For all analyses differences were considered significant at a probability level of 5%.

6.2 Results

6.2.1. Thermal delousing

Statistical analysis revealed a significant increase in the number and percent of vascular lesions at 8 to 9 days after thermal delousing. A significant increase in the number and percent of lamella with hyperplasia was found at both 24-48 hours after and at 8 to 9 days after delousing (Figure 25.). Significantly more fish with lamellar adhesion were observed at eight to nine days after treatment, while significantly fewer fish with lamellar oedema was observed at one day after treatment. However, both lamellar oedema and adhesion was observed in relatively few gills. There was a significant increase the number of fish with epitheliocysts and subepithelial inflammation at both time points after delousing, while there was a significant increase in the number of fish with parasites morphologically consistent with *Trichodina* sp. at 8 to 9 days after delousing. Unicellular parasites morphologically consistent with amoeba associated with segmental hyperplasia was found in 2 fish. Amoebae were not observed in the four fish with positive *N. perurans* qPCR-results. There was a statistically significant increase in the pathogen load of *Ca.* B. cysticola at both time points after thermal delousing, while there was no significant difference in pathogen load for *D. lepeophtherii*. A total of 110 samples were tested for all four putative gill pathogens. Of the 110 samples tested 4 were positive for *N. perurans*, all four positive gill samples were collected 8 to 9 days after delousing. Raw Ct-values ranged from 24.25 to 28.64 (mean 26.98, SE 0.96). Three gill samples were positive for SGPV, with one positive sample per sampling point and positive samples originating from pen A and C. Raw Ct-values ranged from 32.53 to 35.7 (mean 34.39, SE 0.95). Of the 198 samples tested for *Ca.* B. cysticola and *P. theridion*/*D. lepeophtherii* all were positive for *Ca*. B. cysticola, while 196 were positive for *P. theridion/D. lepeophtherii*. Both negative samples were collected before delousing.

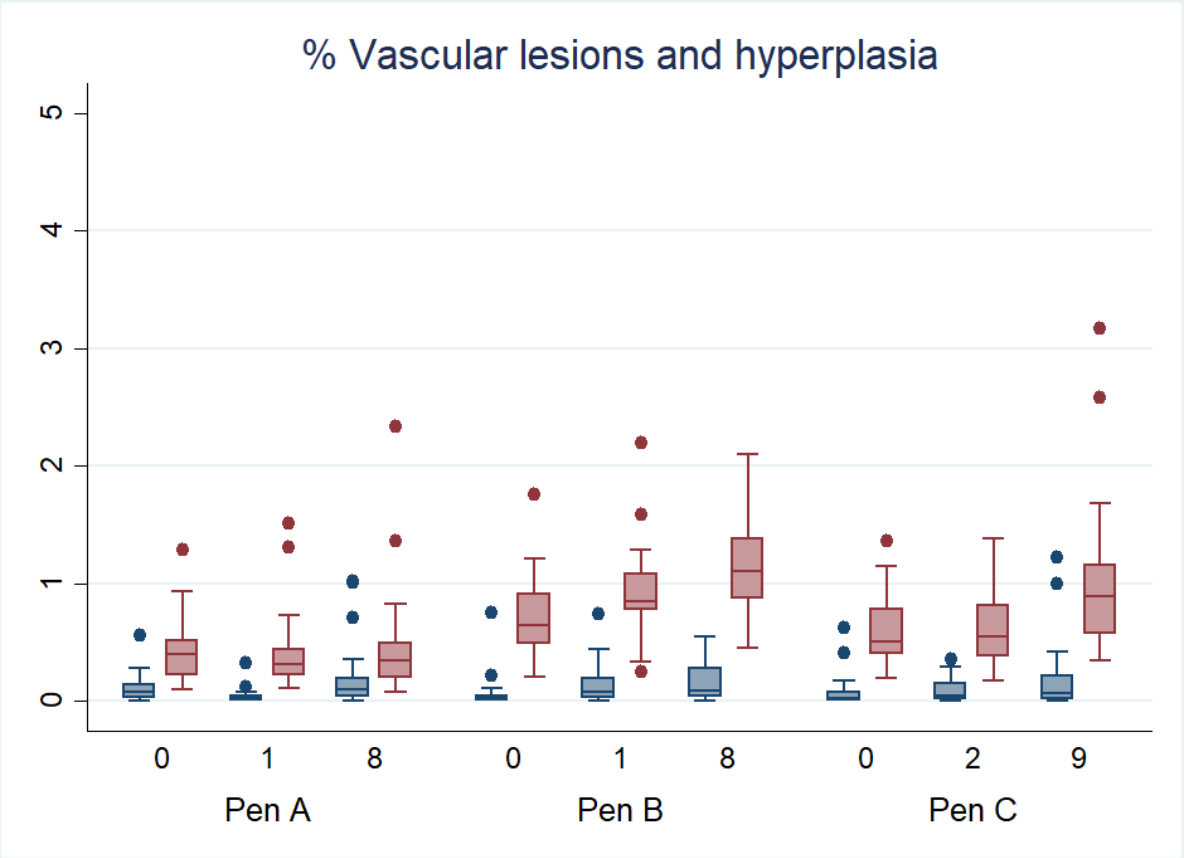


Figure 25. Thermal delousing. Box and whisker plots show percent affected gill tissue per pen and timepoint (day) (n=29-30) for lamellar epithelial hyperplasia (blue) and vascular lesions (red).

6.2.2 Mechanical delousing

Overall, few vascular lesions and foci with hyperplasia were observed in the gills before delousing. Statistical analysis revealed a significant increase in the number and percent of vascular lesions and hyperplasia at 24 to 48 hours and 6 to 7 days after mechanical delousing (Figure 26). Hyperplasia was often found in areas with vascular lesions and likely represent a reactive and reparative response to the vascular damage. There was no clear difference in the number of fish with epitheliocysts or lamellar oedema for the different time points, but there was a slight and statistically significant increase in the number of fish with subepithelial inflammation and *Ichthyobodo* sp. parasites over time.

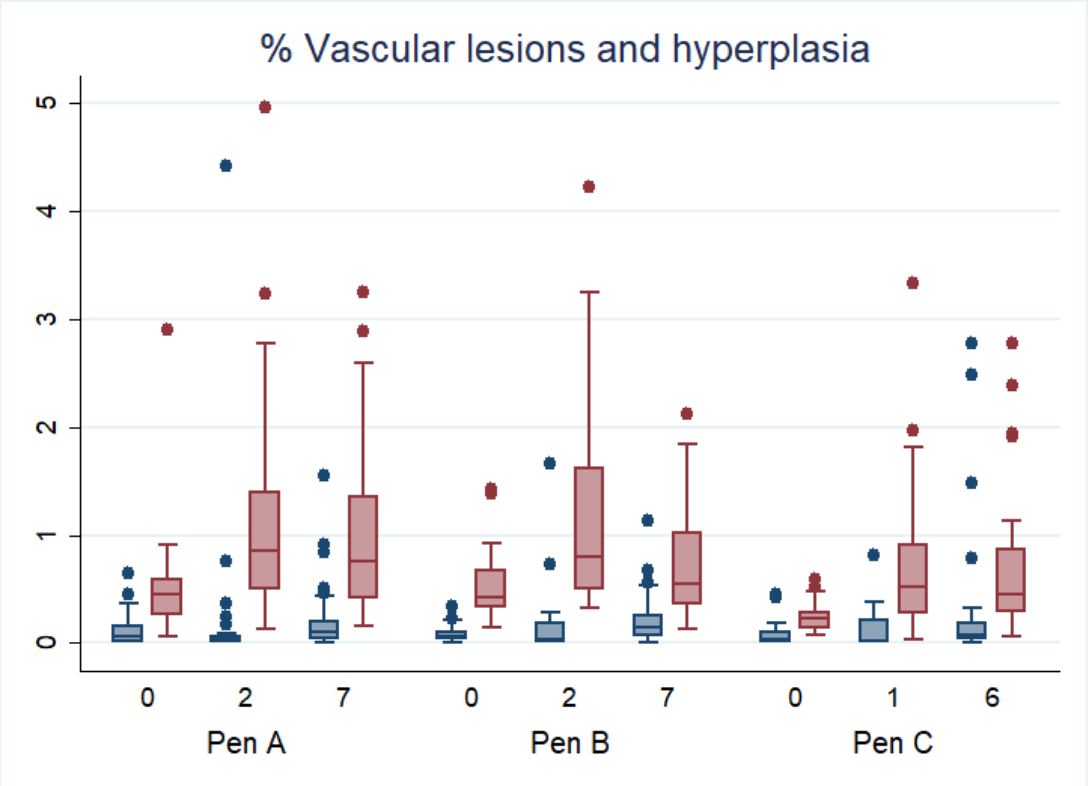


Figure 26. Mechanical delousing. Box and whisker plots show percent affected gill tissue per pen and timepoint (day) (n=30) for lamellar epithelial hyperplasia (blue) and vascular lesions (red).

6.2.3. Net cleaning

In general, very few lesions were observed in the gills both before and after net washing, consistent with overall good gill health. When including all pens in the statistical model there was no statistically significant difference in the number of fish with any of the recorded lesions except for slightly less acute vascular lesions at 1 day post net cleaning. When statistical analysis was repeated for pen A and B there was an increase in the number of fish with subacute vascular lesions, primarily lamellar thrombi, at 1 day after net washing.

8. Discussion

Factors associated with overall mortality

Percent overall mortality in the seawater phase was investigated for three different windows in time. Increased “day degrees in freshwater” and increasing temperatures in seawater were associated with increased mortality during the first 90 days in sea. Increased mortality during the first 180 days at sea was observed in association with increasing seawater temperature and gross gill scores. When percent daily mortality during the full stocking period was the

outcome, associations with increasing sea temperature, gross gill score and delousing events were observed. Overall, the effect of freshwater environment or duration of stay in freshwater (represented here by "day degrees") was only apparent in the earliest part (first 90 days) of the seawater phase. A positive association between gross gill score and percent daily mortality was observed for the first 180 days at sea as well as for the full seawater cycle.

Gill pathology and gill disease in the freshwater phase

In the current study clinical gill disease with gill-related mortality was detected in just one project group at one freshwater site. The affected group suffered from salmon gill poxvirus disease, was supplied from a FT-site, and was sea-transferred in autumn 2018. An additional 3 project groups were infected with SGPV but did not develop clinical disease or mortality. These four SGPV-positive groups were from two separate sites, one with RAS and one FT-systems. Thus, there were no evident differences in whether groups became infected with SGPV between fish kept in RAS or FT-systems or autumn or spring transferred fish. Further, histological examination confirmed that most sampled fish had minimal to mild gill lesions, suggestive of overall good gill health. Due to just one group developing gill disease in the freshwater phase it was not possible to draw any conclusions about differences in prevalence and incidence of gill disease between fish groups depending on water handling systems, timing of sea transfer and environmental or production factors. However, water temperature was positively associated with log-transformed overall daily mortality in freshwater, while the association with CO_2 was negative. The positive association indicates that there were more mortalities when temperatures in the freshwater phase were high. The negative association between high CO_2 levels and total mortality is likely a spurious association as there is no evidence that high levels of CO_2 will have a beneficial impact on fish health.

Gill pathology and gill disease in the marine phase

Gill related mortality, our indicator of clinical gill disease, was relatively low among all project groups and no gill-related mortality was recorded in 6 of 16 project groups. Additionally, gill-related mortality at site H occurred after gross scoring and sampling for laboratory analysis were completed, and as such the exact cause and presence of pathogens and pathology during and prior to this mortality event is unknown. The percent and number of sampled fish with severe gill pathology detected on gross and histological examination were low. Due to this, the results of the association analysis should be interpreted with caution.

In agreement with previous studies there was a very high prevalence of *D. lepeophtherii* and *Ca.* B. cysticola-infection at all eight sea sites, further confirming that these agents are ubiquitous in farmed Atlantic salmon in Western Norway. Salmon gill poxvirus seemed to have a seasonal distribution with positive samples collected in late summer and autumn in most sites. Unsurprisingly there was a clear co-variation and seasonality in the prevalence of *N. perurans* detected with qPCR analysis and the diagnosis of AGD on histology examination.

Detection of amoeba and a diagnosis of AGD on histology was significantly associated with severity of gill histopathology, suggesting that amoeba was contributing to development of gill lesions though the observed effect was small. Our results also showed that a diagnosis of AGD on histology or finding *N. perurans* on qPCR-analysis does not necessarily mean that the fish has severe gill pathology, clinical disease or will have issues with gill related mortality. Further variation in normalized Ct-values for *N. perurans*, *Ca.* B. cysticola, *D. lepeophtherii* or SGPV were not significantly associated with overall mortality, gill-related mortality, or the degree of gill histopathology in this study.

Increasing severity of histopathology lesions were observed with increasing prevalence of amoeba/AGD detected on histology, for fish originating from a RAS-sites, as well as with increasing gross gill scores and decreasing oxygen-levels. These results suggest that fish from RAS may be more susceptible to developing gill disease, as gill histology lesions overall were more severe in fish from RAS-sites, though this association was not replicated when using gill related mortality as an indicator of gill disease. Gill related mortality in the marine phase was only significantly associated with the severity of vascular lesions detected on histopathology. Decreasing oxygen levels is unlikely to directly lead to gill pathology but may serve as a stressor and may make fish more susceptible to infectious disease and impact healing and resolution of tissue lesions once they develop. The association of gross gill scores with histopathology scores was not unexpected as gross gill scores and histology scores are both based on the degree of tissue lesions observed in the gills. This result shows that there was an association between gross and histology observations despite the overall low severity of gill lesions and many different persons performing gross scoring in the study.

Gill health and mortality related to delousing

Potential acute and cumulative effects of delousing on mortality and gill health were assessed in WP1 and WP3. Results from WP3 showed that there was a statistically significant increase in the extent of lamellar epithelial hyperplasia and vascular lesions in gills at one week after mechanical and thermal delousing. In addition, there was an increase in the number of fish with observable putative gill pathogens or lesions presumed to be related to gill pathogens after delousing and an increase in the pathogen density detected by qPCR-analysis for *Ca.* B. cysticola. In the longitudinal study (WP1) delousing episodes involving handling and/or bath treatments were significantly associated with increased overall mortality, suggesting that delousing may also be associated with mortality in fish with relatively good gill health as reflected by generally low gross and histology gill scores and low gill related mortality (for types of delousing operations see Table 1 and Figure 2). Delousing was not significantly associated with increased gill related mortality or with increased severity of gill histopathology when examining data from the longitudinal study. However, further work will be undertaken to detect any associations between delousing operations and histology lesions in this data set.

The current results suggests that thermal and mechanical delousing can have a negative effect on gill health and contribute to development of hyperplastic and vascular lesions in the gill. In

addition, delousing was associated with an acute increase in prevalence and density of gill pathogens suggesting that the treatment may directly or indirectly promote proliferation and/or colonization by these agents. Whether the gill lesions observed will persist or can accumulate over time remains to be determined, but the increase in the percent vascular lesions over time at sea observed in WP1 might suggest that these lesions can accumulate in the gills. It also important to note that it is unknown if the negative effects of delousing on gill health and mortality in the current study are related to the treatment part of the delousing process (i.e., exposure to warm water, water jets or hydrogen peroxide) or other aspects delousing process. As the aim of this study was to detect effects of delousing as it is performed in commercial production, it was not possible to separate the effect of crowding and handling from the specific treatment method used.

Effects of antifouling strategies on gill and overall health

When sampling fish from net pens at one sea site (WP3) before and at one and seven days after net cleaning we found that the number of fish with subacute vascular lesions (thrombi) in the gills at one day after *in situ* net cleaning was significantly increased compared to fish sampled before net cleaning. This negative impact of net cleaning on gill health was small and short lived, as no increase in the number of fish with vascular lesions or other recorded lesions was observed at seven days after net cleaning. There was no observable association between net cleaning and total mortality or gill related mortality in the longitudinal study (WP1). Further, no association between total gill histology score and net cleaning was detected in the preliminary analysis of histology data from the longitudinal study, but further work will be undertaken to detect any associations between net cleaning and histology lesions. While an acute negative impact of net cleaning on gill health was detected the clinical implication of this finding remains to be established. Previous laboratory studies have indicated that exposure to the biofouling organism *Ectopleura larynx* can have a negative impact on gill health (Baxter, et al., 2012; Bloecher, et al., 2018), however no difference in gill lesions after net cleaning was found in a recent field study (Napsøy, 2020).

Phyto- and zooplankton levels in Western Norway

More than 320 water samples analysed for zoo- and phytoplankton in the project showed that zooplankton levels generally were very low during the project period from autumn 2018 to summer 2020. However, fluctuations in plankton levels might have occurred and not been detected due to the weekly to monthly water analysis schedule. The highest levels of phytoplankton and zooplankton was found during spring, and the dominant phytoplankton observed were from the genus *Pseudo-nitzschia* and other diatoms. While there were some variations in the phytoplankton levels, there was no clear co-variation between plankton levels and gross or histology gill scores nor was gill-related mortality recorded following the peak plankton levels. Further, the plankton levels were neither associated with variation in

total histology score, gill-related mortality nor overall mortality. Thus, our results suggest that zoo- and phytoplankton levels in Western Norway in 2018-2020 were generally low. Further, the plankton levels and types of phytoplankton observed in this study did not lead to gill pathology or gill-related mortality.

Serum biochemistry as an on-site non-lethal indicator of gill function

The use of serum biochemistry on-site requires the availability of user-friendly and reliable POC-device that can be used in the field. When testing the Vetscan iSTAT POC-device we found that this analyzer was not a reliable tool for assessment of the electrolyte's chloride, sodium, or potassium in Atlantic salmon. This is in line with earlier results in other fish species. Harter et al. (Harter, et al., 2014) attempted to validate the iSTAT system on rainbow trout and found that haematocrit and parameters sodium, partial pressure of carbon dioxide, partial pressure of oxygen and bicarbonate were not reliably measured. Similarly, in a study of cod (*Gadus morhua*), comparing the iSTAT with conventional laboratory techniques, it was found that the POC-device was inaccurate for measurement of pH, pO_2, haematocrit, sodium, potassium, calcium, and haemoglobin (Borissov, et al., 2019). In the current study there was a good agreement between the iSTAT and the converted Pentra C400 values for lactate, though values from the POC-device cannot be directly compared to plasma values from the laboratory analyzer because they use different source materials (whole blood versus plasma).

Further, we did not find that mild gross and histological gill lesions were associated with changes in blood sodium, potassium, lactate, glucose or PCO_2, although PCO_2 levels were measured using the iSTAT and could be unreliable. Elevated levels of lactate, chloride, sodium, and potassium have been reported in fish with AGD (Hvas, et al., 2017), and the lack of clinical gill disease and moderate to severe pathology in the fish sampled in the current project could possibly explain this discrepancy. However, the use of clinical chemistry parameters and blood gas as non-lethal indicators of gill disease in the field could be problematic due to issues with sampling and the non-specificity of the measured analytes. Levels of blood gas will be impacted by the anaesthesia necessary for humane handling and sampling of the fish, and thus it will be challenging to interpret the results of analysis in a field setting. Also, while changes in electrolytes and lactate levels may occur in cases of gill disease, these analytes are not specific for gill disease (Iversen, et al., 2005; Olsen, et al., 1992; Rodger, et al., 1991). As such clinical chemistry could be helpful to assess the impact of gill lesions and gill disease on fish physiology but the method cannot alone be used to confirm a diagnosis of gill disease.

9. Deliverables

8.1 Reports

- Rapport arbeidspakke 2 (Norwegian) – Appendix file 6.

8.2 Presentations, posters, and other publications

- "Evaluation of a gross "total" gill score against a standardized histology score and qPCR in farmed Atlantic salmon (*Salmo salar*)", poster EAFP conference, Porto, 2019 – Appendix file 7.
- «Hva styrer hvor mye lus man har på anlegget? Overlevelse, spredning, behandlingseffekt. Og litt nytt fra Gillrisk (FHF 901515)», oral presentation Lusekonferansen, Trondheim January 2021.
- «Effekt av termisk avlusning på gjellehelse», oral Presentation Havbruk 2020
- Webinar «Gjellehelse hos oppdrettslaks – foreløpige resultater fra Gillrisk-prosjektet», Oslo, April 2021
- "Prevalence and temporal development of salmon gill poxvirus infection in Atlantic salmon (*Salmo salar* L) from freshwater to seawater", poster Gill Health Initiative 2021 – Appendix file 8.
- "Acute effects of mechanical delousing on gill health of farmed Atlantic salmon (*Salmo salar*)", oral presentation Gill Health Initiative 2021
- Popular scientific article on impact on thermal and mechanical delousing on web news site fish health forum, https://fishhealthforum.com/non-medicinal-delousing-approaches-cause-damage-to-salmon-gill-tissue/
- "GillRisk: En kohort-studie om gjelleinfeksjoner, gjellepatologi og gjelle-relatert dødelighet hos norsk oppdrettslaks", oral presentation Frisk fisk 2022

8.3 Scientific publications

- "Evaluation of agreement between a laboratory based and a field-based blood analyser for analysis of selected biochemical analytes in farmed Atlantic Salmon (*Salmo salar* L.)". https://eafp.org/download/2021-volume41/issue_1/41-1-17-ostevik.pdf
- "Assessment of acute effects of *in situ* net cleaning on gill health of farmed Atlantic salmon (*Salmo salar* L.)". https://doi.org/10.1016/j.aquaculture.2021.737203
- "Effects of thermal and mechanical delousing on gill health of farmed Atlantic salmon (*Salmo salar* L.)". https://doi.org/10.1016/j.aquaculture.2022.738019
- "A cohort study of gill infections, gill pathology and gill-related mortality in sea farmed Atlantic salmon (*Salmo salar* L.): Descriptive analysis". https://doi.org/10.1111/JFD.13662
- A fifth scientific article is in currently in progress. Tentative title: "A cohort study of gill infections, gill pathology and gill-related mortality in sea farmed Atlantic salmon (*Salmo salar* L.): Epidemiological analysis"

10. Appendixes

Appendix 1. Overview of project samples

Appendix 2. Protokoll prøveuttak zoo- og phytoplankton (in Norwegian)

Appendix 3. Makroskopisk gjellescoring (in Norwegian)

Appendix 4. Variables for association analysis and univariable analysis results

Appendix 5. Rapport arbeidspakke 2 (in Norwegian)

Appendix 6. Poster: Evaluation of gross gill score against a standardized histology score

Appendix 7. Poster: Prevalence and temporal development of salmon gill poxvirus infection in Atlantic salmon

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APPENDIX 1

Group /	A		B		C		D		E		F		G		H	
Sampling no.	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		30		0		0		0	
Sum FW	**60(h) 59(p)**		**NS**		**NS**		**60**		**50 (h) 79 (p)**		**60**		**60**		**60**	
4	20	NS	20	20	10	10	20	NS	30	30	30	30	30	30	30	30
5	20	20	20	20	20	20	20 (h) 19 (p)	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	30	25	20	30	30	30	30
8	30	30	30	30	30	30	30	30	30 (h) 27 (p)	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**

Table 1. 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	A1	A2	B1	B2	C1	C2	D1	D2	E1	E2	F1	F2	G1	G2	H1	H2
#Fish	**1089**	**1047**	**971**	**997**	**922**	**1055**	**1015**	**1029**	**747**	**746**	**1006**	**985**	**921**	**919**	**1091**	**1013**
#Weeks	**51**	49	46	47	43	49	49	49	35	33	45	44	43	43	50	**47**

Table 2. Sample overview gross scores. Total number of fish scored and weeks with gross scores available per project group. Gross scoring was only done during the sea phase of production.

APPENDIX 2

FishVet Group

Prøvetaking for fytoplankton

Utstyr

- Fytoplankton håv (25 cm i diameter)
- Spruteflaske fylt med sjøvann
- Prøveflaske (300ml, hvit)
- Hansker
- Lugols jodløsning
- Pipette, engangs
- Rekvisisjonsskjema
- Vannfast penn
- Aluminiumsfolie

FishVet Group

Prøvetaking for fytoplankton

1. Bruk den minste håven til fytoplankton. **Senk håven sakte ned til 10 meter. Trekk håven langsomt opp** (ikke raskere enn 0,5m/sek, bruk knutene på tauet (1 knute per meter)).		4. Spyl igjennom 300ml med sjøvann for å overføre prøven til flaska.		7. Hver 4. uke, send inn de 4 vannprøvene for fytoplankton sammen med 4 vannprøver for zooplanktonanalyse. Legg ved utfylt rekvisisjonsskjema og bruk vedlagte returetiketter.
2. Når håven er ved overflaten, hev og senk nettet langsomt uten at det kommer helt under vann, slik **at prøvematerialet kommer ned i bunnen til filteret**. Løft nettet ut av vannet og **spyl sjøvann ned på sidene** for å få med mest mulig materiale.		5. Tilsett ca 5 ml Lugols jodløsning til flaska slik at fargen på prøven ligner svak te. Sørg for at flaska er merket med dato og lokalitet.		8. Skyll håven og filteret godt i rent vann og heng til tørk.
3. Skru av filteret på bunnen av håven, legg det opp ned på beholderen for fytoplankton (tom hvit plastbeholder merket med grønt kryss).		6. Pakke vannprøver i aluminiumsfolie. Lagre mørkt, helst i kjøleskap.		

FishVet Group

Prøvetaking for zooplankton

Utstyr

- Zooplankton håv (50 cm i diameter)
- Sprute flaske fylt med sjøvann
- Prøvebeholder (formalin), med rødt kryss
- Hansker
- Rekvisisjonsskjema
- Vannfast penn
- Secchidisk

FishVet Group

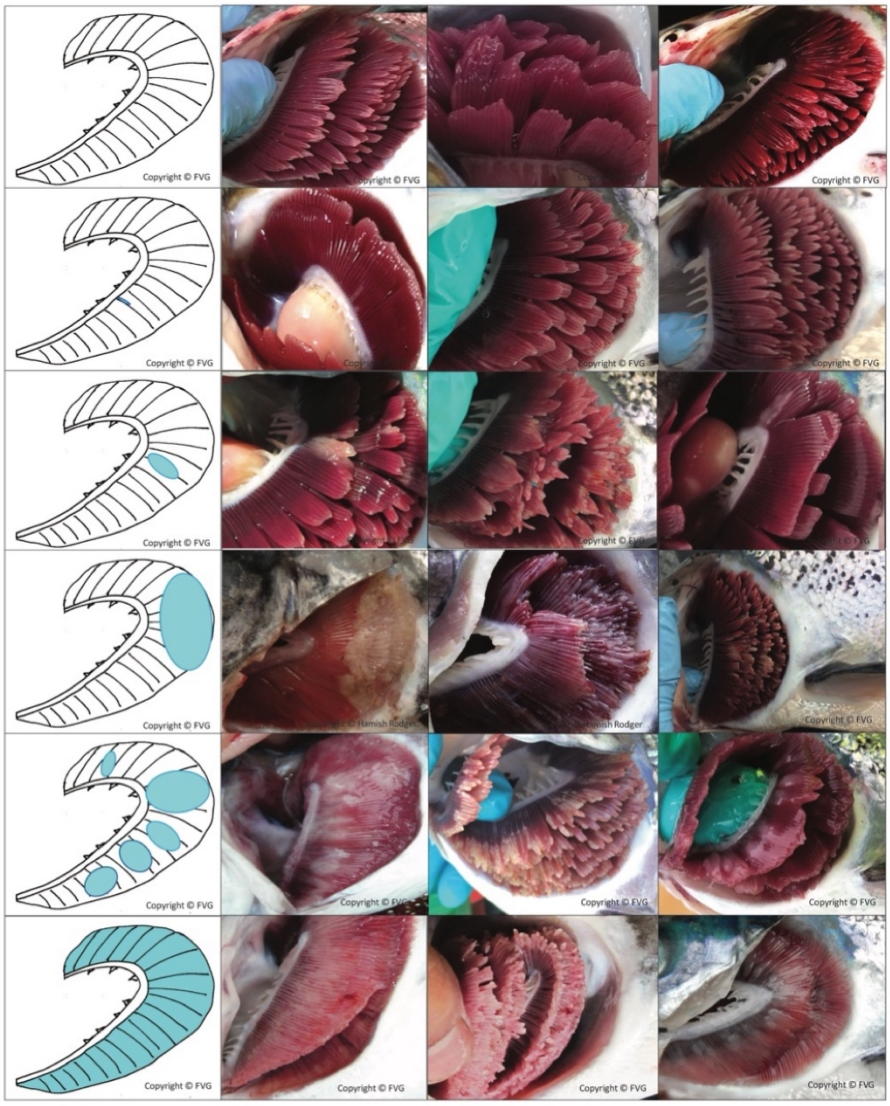
Prøvetaking for zooplankton

1. Secchidisk-avlesning: Senk secchidisken ned i vannet til disken ikke lenger er synlig. Hev disken langsomt til du kan se tydelig forskjell mellom de hvite og svarte delene av disken – noter denne dybden.		4. Skru adapteren på formalinbeholderen og plasser filteret på adapteren.		7. Send inn vannprøve hver 4. uke sammen med utfylt rekvisisjon. For forsendelse bruk returetiketter sendt sammen med utstyr for prøvetaking.
2. Bruk den største håven til zooplankton. Sjekk at håven er uskadet og at filteret er godt skrudd på. Senk håven sakte ned til 10 meter. Trekk håven langsomt opp (ikke raskere enn 0,5m/sek, bruk knutene på tauet (1 knute per meter)).		5. Spyl med 150 milliliter sjøvann for å overføre prøven til beholderen. (Totalvolum i beholderen skal tilslutt være 300 ml).		8. Skyll håven og filteret godt i rent vann og heng til tørk
3. Når håven er ved overflaten, hev og senk nettet langsomt uten at det kommer helt under vann, slik at prøvematerialet kommer ned i bunnen til filteret. Løft nettet ut av vannet og spyl sjøvann ned på sidene med sprute flasken for å få med mest mulig materiale.		6. Sørg for at beholderen er merket med dato og lokalitet		

APPENDIX 3

Gjellescore – GILLRISK
Prosjekt FHF 901515

FishVet Group



Prosjekt FHF 901515: Risikofaktorer, indikatorer og strategisk håndtering av gjellelidelser hos atlantisk laks (GILLRISK). GillRisk Gjellescore

1. GillRisk er en totalscore = alle skader på gjellen som fører til redusert gjellefunksjon.
2. Ha en lyskilde tilgjengelig. En hodelykt med godt lys anbefales.
3. Scor hver gjellebue på venstre side separat og noter i score-skjemaet
5. Noter hvilke type(r) forandringer du ser funn gjelle-feltet, bruk kodene listet opp under.
6. Forandringer/skader som ikke er beskrevet i tabell. Noter i kommentarfeltet, beskriv og ta gjerne bilde
7. Noen forandringer skal ikke scores, men kodes i funn-feltet(se tabellen) under.

Acknowledgement: All pictures provided by Dr. Hamish Rodger, Angela Ashby and Chris Matthews.

Gjellescore – GILLRISK

Forandringer gjelle	Kode
Hvite flekker med og uten slim (AGD)	**H**
Blødning	**BØ**
Gul misfarging/områder på gjelle (filamenttupper)	**GU**
Nekrose (grått, dødt vev)	**N**
Svinn/tap eller forkortede filamenter	**SV**
Svulne ,fortykkede filamenter	**FF**
Sammenlodning filamenter	**SF**
Funn, score ikke men noteres i funn-feltet	
Røde/blodfylte gjeller (ikke blødning)	RØ
Skade gjellebue (sår, misfarging, blødning)	GB
Forøket mengde slim	SØ
Skade/misdannelse gjellelokk	GL
Bleke gjeller (hele gjellen)	BK
Respirasjonsproblem (gisping etter luft, økt gjellelokkfrekvens, utspilte gjellelokk)	RP

H – gråhvite fortykkede filamenter
SF- sammenlodning filamenter

SØ – forøket mengde slim

BØ – blødning
BK- bleke gjeller

H – hvite flekker

H – hvite flekker

GU - gule områder
(filamenttupper)

N - nekrose (dødt vev)

SV– svinn, forkortede
filamenter

B – bleke gjeller (hele
gjellen)

R – røde/blodfylte gjeller

GB – skade gjellebue

BØ - blødning

Acknowledgement: All pictures provided by Dr. Hamish Rodger, Angela Ashby and Chris Matthews.

APPENDIX 4

Appendix of variables for association analysis and output of the univariable analysis

Variable name	Stage	Origin of variable	Description
Amoeba	SW	Project samples	Histology samples where amoeba was observed, then aggregated as a percent on group level. The value has been repeated for 14 days.
Arthropods	SW	Project samples	Histology samples where arthropods was observed, then aggregated as a percent on group level.
Ca.B.Cysticola in fw	FW	Project samples	Presence of *Ca.B.Cysticola* detected on qPCR at some point during the freshwater cycle. Yes/no variable.
Day degrees in fw	FW	Production data	Aggregated daily temperature in the freshwater production cycle
Days with CO_2 above 15	FW	Production data	Count of days with CO_2 values above 15mg/l
Deformed gills	FW	Project samples	Histology samples where deformed gills was observed, included as a binary value of presence/absence in the group before transfer to sea.
Delousing week	SW	Production data	Yes/no, Non-feed delousing has taken place. Variable is kept as 1 for the week following a delousing operation.
Epitheliocysts	SW	Project samples	Histology samples where epitheliocysts was observed, then aggregated as a percent on group level. The value has been repeated for 14 days.
FW total mortality	FW	Production data	Total percent mortality during the freshwater period.
Gross score POL	SW	Collected by site staff as a part of project	Weekly gross score values, used a linear interpolation to get daily values by using "mipolate" in Stata.
K-factor	FW	Condition factor estimated before transfer to sea	The group condition factor being the average of individual condition factor estimates calculated: weight*0,1/(length1*0,1)^3
medianAGDCTnormPOL	SW	Project samples	Normalized median Ct-values for *N. perurans*
medianBcysCTnormPOL	SW	Project samples	Normalized median Ct-values for *Ca.B.Cysticola*
medianPOXCTnormPOL	SW	Project samples	Normalized median Ct-values for SGPV
medianPtheCTnormPOL	SW	Project samples	Normalized median Ct-values for *P.Theridion /D.lepeophtherii*
moves in fw	FW	Production data	Number of moves during the freshwater period
Net cleaning	SW	Production data	Yes/no, Net cleaning has taken place at site. Variable is kept as 1 for three days following a net cleaning operation.
Oxygen	SW	Production data	Sea oxygen levels registered by sea site staff
Pox + PCR in fw (all test)	FW	Project samples and routine health samples	Yes/no variable if the group tested positive for SGPV during the freshwater period
Pox + PCR in fw (Project test)	FW	Project samples	Yes/no variable if the group tested positive for SGPV during the freshwater period
RAS	FW	Information from producer	RAS or flow-through freshwater site
S0 vs S1	FW	Production data	Fish put to sea in autumn (S0) or spring (S1)
Sea temperature	SW	Production data	Daily seawater oxygen levels
Weight spread coef.	FW	Weight spread coefficient before transfer to sea	The group weight spread factor calculated as the (SD weight/average weight)*100
Total histology score	SW	Project samples	The total histology score on group level. The value has been repeated for 14 days post sampling date.
Vascular lesions	SW	Project samples	Percent of gill tissue with vascular lesions detected on histology, aggregated as the median on group level. The value has been repeated for 14 days.
Vax PD7 vs m6	FW	Production data	Whether the fish group was vaccinated with Aquavac PD7 vet. or ALPHA JECT micro 6 vaccine.
Winter signal	FW	Information from producer	Some freshwater facilities use 24h light throughout the freshwater production cycle, other give the fish a winter signal (i.e. darkness). Yes/no variable.

Outcome total mortality, 90d into seawater phase (log-transformed)

	Variable tested	#Observations	Groups, level 1	Groups, level 2	Coef.	P	[95% Conf. Interval]	
FW	RAS	1290	8	16	-0.0791	0.84	-0.8298	0.6716
	S0 vs S1	1290	8	16	0.1844	0.63	-0.5574	0.9262
	moves in fw	1290	8	16	0.0538	0.62	-0.1566	0.2641
	FW total mortality	1290	8	16	-0.0866	0.4840	-0.3292039	0.1559566
	Winter signal	1290	8	16	-0.2548	0.49	-0.9859	0.4762
	Day degrees in FW	1290	8	16	0.0008	0.20	-0.0004	0.0019
	Days with CO2 above 15	1290	8	16	0.0095	0.07	-0.0009	0.0198
	Weight spread coef.	1290	8	16	-5.8902	0.00	-9.6242	-2.1561
	K-factor	1290	8	16	-0.0581	0.14	-0.1348	0.0186
	Vax PD7 vs m6	1290	8	16	0.1927	0.73	-0.8919	1.2772
	Pox + PCR in fw (GR test)	1290	8	16	omitted			
	Pox + PCR in fw (all test)	1290	8	16	-0.2611	0.48	-0.9912	0.4689
	Ca.B.Cysticola in fw (GR test)	1290	8	16	0.5422	0.32	-0.5313	1.6156
	Sea temperature	1291	8	16	0.1409	0.00	0.1084	0.1734
SW	Oxygen	524	7	14	0.0035	0.68	-0.0130	0.0199
	Arthropods	556	8	16	0.0914	0.89	-1.2499	1.4328

Outcome total mortality, 180d into seawater phase (log-transformed)

	Variable tested	#Observations	Groups, level 1	Groups, level 2	Coef.	P	[95% Conf. Interval]	
	RAS	2620	8	16	0.0576	0.80	-0.3906	0.5057
	S0 vs S1	2620	8	16	0.2780	0.18	-0.1287	0.6847
	moves in fw	2620	8	16	0.0538	0.62	-0.1566	0.2641
	FW total mortality	2620	8	16	-0.0339	0.65	-0.1816	0.1137
	Winter signal	2620	8	16	-0.3403	0.08	-0.7233	0.0427
	Day degrees in FW	2620	8	16	0.0006	0.04	0.0000	0.0013
FW	Days with CO2 above 15	2620	8	16	0.0075	0.01	0.0023	0.0127
	Weight spread coef.	2620	8	16	-0.0173	0.50	-0.0677	0.0331
	K-factor	2620	8	16	-2.7122	0.05	-5.4425	0.0181
	Vax PD7 vs m6	2620	8	16	-0.3346	0.11	-0.7431	0.0739
	Pox + PCR in fw (GR test)	2620	8	16	omitted			
	Pox + PCR in fw (all test)	2620	8	16	-0.1481	0.51	-0.5862	0.2900
	Ca.B.Cysticola in fw (GR test)	2620	8	16	1653296.0000	0.63	-0.5054	0.8360
	Sea temperature	2620	8	16	0.0770	0.00	0.0636	0.0905
	Oxygen	1053	8	16	0.0052	0.30	-0.0045	0.0148
SW	Epitheliocysts	748	8	16	0.0011	0.25	-0.0008	0.0031
	Amoeba	748	8	16	0.0004	0.83	-0.0032	0.0040
	Gross score POL	1886	8	16	0.7467	0.00	0.4738	1.0197

Outcome total mortality in the whole seawater phase (log-transformed)

	Variable tested	#Observations	Groups, level 1	Groups, level 2	Coef.	P	[95% Conf. Interval]	
FW	RAS	7635	8	16	0.1663	0.42	-0.2390	0.5716
	S0 vs S1	7635	8	16	0.1254	0.55	-0.2869	0.5376
	FW total mortality	7635	8	16	-0.0788	0.23	-0.2077	0.0501
	Moves in fw	7635	8	16	-0.0786	0.42	-0.2711	0.1139
	Winter signal	7635	8	16	-0.1401	0.50	-0.5500	0.2699
	Day degrees in FW	7635	8	16	-0.0001	0.71	-0.0008	0.0006
	Days with CO2 above 15	7635	8	16	-0.0001	0.98	-0.0053	0.0052
	Weight spread coef.	7635	8	16	0.0136	0.58	-0.0340	0.0612
	K-factor	7635	8	16	0.9612	0.54	-2.0734	3.9958
	Vax PD7 vs m6	7635	8	16	-0.0980	0.66	-0.5358	0.3397
	Pox + PCR in fw (GR test)	7635	8	16	-0.0836	0.70	-0.5148	0.3476
	Pox + PCR in fw (all test)	7635	8	16	0.0834	0.70	-0.3339	0.5008
	Ca.B.Cysticola in fw (GR test)	7635	8	16	0.1331	0.68	-0.4977	0.7639
SW	Delousing week	7635	8	16	1.2191	0.00	1.1213	1.3169
	Net cleaning	7635	8	16	0.4026	0.00	0.2397	0.5656
	Sea temperature	7633	8	16	0.0751	0.00	0.0663	0.0840
	Oxygen	3018	8	16	-0.0235	0.00	-0.0279	-0.0191
	Epitheliocystis	1874	8	16	-0.0007	0.41	-0.0023	0.0009
	Amoeba	1874	8	16	0.0020	0.17	-0.0009	0.0049
	Gross score	6039	8	16	1.0774	0.00	0.9839	1.1710

Outcome GILL mortality in the whole seawater phase Neg. Binomial reg

	Variable tested	#Observations	Groups, level 1	Groups, level 2	Coef.	P	[95% Conf. Interval]	
FW	RAS	8,329	8	16	-3.5212	0.30	-10.1677	3.1252
	S0 vs S1	8,329	8	16	4.6413	0.16	-1.7866	11.0693
	Moves in fw	8,329	8	16	2.4787	0.12	-0.6246	5.5821
	Winter signal	8,329	8	16	3.6927	0.28	-2.9327	10.3180
	K-factor	8,329	8	16	53.2161	0.01	10.7515	95.6807
	Weight spread coef.	8,329	8	16	0.6356	0.10	-0.1183	1.3895
	Days with CO2 above 15	8,329	8	16	-0.0889	0.10	-0.1934	0.0156
	Day degrees in FW	8,329	8	16	-0.0081	0.13	-0.0185	0.0023
	Vax PD7 vs m6	8,329	8	16	-13.9695	0.12	-31.7802	3.8412
	Pox + PCR in fw (GR test)	8,329	8	16	omitted			
	Pox + PCR in fw (all test)	8,329	8	16	doesn't work			
	Ca.B.Cysticola in fw (GR test)	8,329	8	16	5.8589	0.37	-6.8981	18.6160
SW	Delousing week	8,329	8	16	-0.1869	0.58	-0.8524	0.4786
	net cleaning	8,329	8	16	-1.3250	0.02	-2.4464	-0.2037
	Total histology score	1915	8	16	0.0422	0.00	0.0180	0.0664
	Vascular lesions	1915	8	16	0.4430	0.00	0.3058	0.5802
	Median POX CTnorm	6204	8	16	2187.7240	0.04	53.7975	4321.6510
	Median Bcys CTnorm	6204	8	16	-36.7950	0.00	-48.2340	-25.3560
	Median AGD CTnorm	6204	8	16	-10.6639	0.32	-31.6284	10.3006
	Median Pthe CTnorm	6204	8	16	-401.1136	0.00	-480.3993	-321.8280
	Sea temperature	8327	8	16	0.4931	0.00	0.3994	0.5869
	Oxygen	3197	8	16	-0.0247	0.09	-0.0534	0.0041
	Epitheliocysts	2050	8	16	-0.0158	0.00	-0.0247	-0.0068
	Amoeba	2050	8	16	-0.0390	0.00	-0.0574	-0.0206
	Gross score	6582	8	16	1.1354	0.00	0.6090	1.6618

Outcome median total histoscore pulled 14 days, log-transformed								
	Variable tested	#Observations	Groups, level 1	Groups, level 2	Coef.	P	[95% Conf.	Interval]
FW	Pox + PCR in fw (all test)	1921	8	16	-0.0696	0.71	-0.4425	0.3032
	RAS	1920	8	16	0.2969	0.07	-0.0180	0.6119
	S0 vs S1	1921	8	16	0.0019	0.24	-0.0013	0.0051
	Winter signal	1920	8	16	0.2828	0.08	-0.0383	0.6039
	Days with high CO2	1920	8	16	-0.0003	0.92	-0.0065	0.0058
	PD7 vs M6	1011	8	16	0.4723	0.21	-0.2728	1.2173
	Deformed gills	131	6	12	omitted			
	Day degrees in fw	1921	8	16	0.0000	0.89	-0.0007	0.0006
SW	Delousing week	1921	8	16	0.3319	0.00	0.1436	0.5201
	Net cleaning	1921	8	16	0.3627	0.01	0.0922	0.6332
	Median POX CTnorm	1735	8	16	275.0747	0.00	125.0778	425.0716
	Median Bcys CTnorm	1735	8	16	0.8337	0.51	-1.6726	3.3399
	Median AGD CTnorm	1735	8	16	28.8489	0.00	23.3144	34.3834
	Median Pthe CTnorm	1735	8	16	53.4853	0.00	44.5915	62.3789
	Gross score	1828	8	16	1.4009	0.00	1.2753	1.5265
	Sea temperature	1918	8	16	-0.0141	0.08	-0.0300	0.0019
	Oxygen	740	8	16	-0.0404	0.00	-0.0481	-0.0327
	Epitheliocysts	1921	8	16	-0.0003	0.68	-0.0017	0.0011
	Amoeba	1921	8	16	0.0249	0.00	0.0226	0.0272

APPENDIX 5

Rapport Arbeidspakke 2: Serum biokjemiske og blodgass parametre som potensielle indikatorer for gjellefunksjon og gjellehelse

Oppsummering

Formålet med arbeidspakke 2 var å identifisere operative måleindikatorer for gjellehelse og gjellefunksjon som kan brukes på «merdkanten» for å vurdere behovet for behandling eller tiltak fokusert på bedring av gjellehelsen og/eller forutsi fiskens evne til å tolerere stressende håndtering/behandling. Utvalgte blodparameter skulle evalueres som potensielle indikatorer for gjellefunksjon. En bærbar point-of-care enhet for blodanalyse skulle testes og sammenlignes med en laboratorieenhet for å vurdere pålitelighet og nytteverdi av de ulike analysene. Blodparametrene natrium, klorid, kalium, laktat, blodgasser; skulle korreleres med histologisk og makroskopisk gjellescore for å identifisere mulige parameter som reflekterer redusert gjellehelse og gjellefunksjon. Det ble uttrykt bekymringer om den planlagte bruken av den håndholdte enheten iSTAT (Abaxis) fra FHF og referansegruppen, ettersom erfaringer har vært at iSTAT ikke har fungert optimalt for fisk. Det ble undersøkt om det fantes andre enheter som kunne brukes i stedet for iSTAT, men vi fant ingen andre håndholdte enheter som kunne måle alle de ønskede analyttene.

Ettersom det var tvil om påliteligheten til iSTAT ble det gjennomført to pilotstudier (se detaljert rapport på engelsk). I den første studien ble 21 fisk på omtrent 200 gram uten gjellesykdom avlivet og blodprøver ble tatt umiddelbart etter avlivning. Heparinisert fullblod ble brukt til iSTAT-analysen, mens resterende blod ble sentrifugert og plasma ble fryst i eppendorfrør frem til analyse på Pentra C400 ved laboratoriet. Samtlige verdier for urea målt av iSTAT var lavere enn 1,0 mmol/l og under nedre grenseverdi for iSTAT slik at meningsfull sammenligning med laboratorie-enheten og statistisk analyse ikke var mulig. Statisk analyse var heller ikke mulig for kalium eller klorid, ettersom majoriteten av verdiene var utenfor den

håndholdte enhetens grenseverdi. For laktat gav iSTAT verdier for samtlige fisk, og korrelasjonen mellom laboratorie-enheten og den håndholdte enheten for denne parameteren var god (0.95), men konkordans koeffisienten var relativt lav ved direkte sammenligning mellom enhetene (0.49). Ved sammenligning mellom konverterte laktatverdier (C = 1– hematokritt) fra lab-enheten og den håndholdte enheten var konkordansen god (0.91).

Den andre piloten ble gjennomført på en sjølokalitet på Nord-Vestlandet der fisken hadde hatt synlig gjellepatologi noen uker tidligere. Etter avlivning ble det gjort makroskopisk gjellescoring, blodprøvetaking og uttak av gjellevev til histologi. Både samsvar mellom den håndholdte enheten og laboratorie-enheten og eventuell assosiasjon mellom gjellescore og biokjemiske parameter ble vurdert. Statisk analyse ikke mulig for klorid eller urea, ettersom majoriteten av de målte verdiene var utenfor den håndholdte enhetens grenseverdi. Det var dårlig samsvar (konkordans) og korrelasjon mellom verdiene målt på laboratorie-enheten og den håndholdte enheten for natrium og kalium. Grafisk fremstilling av gjellescore og blodanalytter (Box and whiskers plot) viste ingen tydelig sammenheng mellom gjellescore analyttverdi for urea, laktat, natrium, kalium, klorid, eller totalt karbondioksid. Majoriteten av fiskene hadde lav histologisk og makroskopisk gjellescore, så mangelen på sammenheng kan skyldes at gjellefunksjonen hos disse fiskene var relativt god.

Oppsummert viste pilotene at iSTAT ikke fungerer bra for analyse av urea, klorid, kalium eller natrium i blodet hos atlantisk laks. Det er god korrelasjon mellom nivået av laktat målt av den håndholdte-enheten og laboratorie-enheten. Det ble ikke funnet noen sikker sammenheng mellom makroskopisk eller histologisk gjellescore og nivået av laktat, kalium, natrium, klorid, urea eller totalt karbondioksid i blodet hos atlantisk laks. På grunnlag av disse negative resultatene og ettersom hovedmålet med arbeidspakke 2 var å finne operative måleindikatorer for gjellehelse og gjellefunksjon kunne brukes på «merdkanten» ble

det avgjort denne arbeidspakken av avsluttes. Biokjemisk analyse av blod og undersøkelse av blodgassnivåer har fortsatt nytteverdi for å evaluere gjellefunksjon, men pilotene våre indikerer at iSTAT ikke kan brukes til dette og at blodgass og biokjemisk blodanalyse ikke er praktiske for å evaluere gjellehelse i klinisk praksis.

APPENDIX 6

Evaluation of a gross "total" gill score against a standardized histology score and qPCR in farmed Atlantic salmon (*Salmo salar*)

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Introduction: Gross gill scoring is widely used for monitoring of amoebic gill disease, however a gill score system encompassing all gill lesions likely associated with reduced gill health may provide useful information when making decisions with regards to treatment and other management operations. We report preliminary results on agreement between newly developed gross and histologic gill scoring systems and qPCR-results for selected gill pathogens.

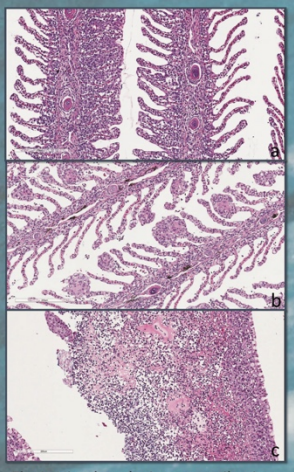

Fig.1. Histology lesions contributing to the total scores. a) Hyperplasia, b) vascular lesions (thrombosed vessels), c) necrosis and loss of secondary lamella

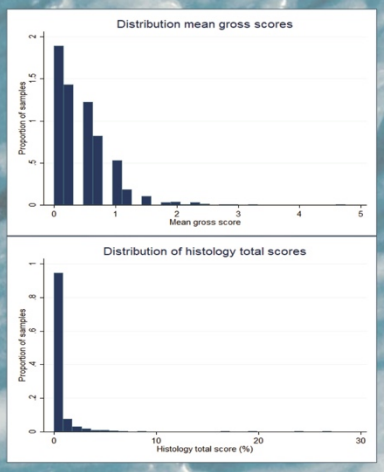

Fig.2. Mean gross scores (top) and histology total scores (bottom). Distribution of scores are skewed to the left with very few gills receiving medium or high scores.

Materials and Methods:

- Samples from 4 sea farms in western Norway
- Data from a longitudinal field study on gill health
- Gross scores from 0 to 5, based on the area of gill tissue deemed to be abnormal
- Histology total scores given as percentage of secondary lamella affected by hyperplasia and/or inflammation, vascular lesions and necrosis (Fig. 1.)
- Presence or absence of pathogens recorded
- qPCR: Salmonid poxvirus, *Neoparamoeba perurans*, *Desmozoon lepeophtherii* and *Candidatus* Branchiomonas cysticola
- Pearson's correlation coefficient calculated to assess agreement

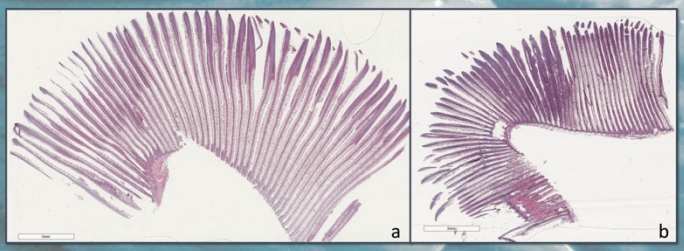

Fig.3. Histology image of gills of assigned a total gill score of a) 0% and b) 19, 4% representing the minimum and maximum area of gill tissue affected by lesions.

PCR-results /Sites		Site A	Site B	Site C	Site D	Total
N. perurans	Negative	277 (99%)	219 (73%)	202 (72%)	199 (71%)	897 (79%)
	Positive	3 (1%)	81 (27%)	78 (28%)	80 (29%)	242 (21%)
Salmonid pox virus	Negative	279 (99,6%)	259 (86%)	260 (93%)	272 (97%)	1070 (94%)
	Positive	1 (0,4%)	41 (14%)	20 (7%)	7 (3%)	69 (6%)
D.lepeophtherii	Negative	28 (10%)	6 (2%)	12 (4%)	0 (0%)	46 (4%)
	Positive	252 (90%)	294 (98%)	268 (96%)	279 (100%)	1.093 (96%)
Ca.B.cysticola	Negative	10 (4%)	88 (29%)	34 (12%)	65 (23%)	197 (17%)
	Positive	270 (96%)	212 (71%)	246 (88%)	214 (77%)	942 (83%)
Total no. samples		280	300	280	279	1.139

Table 1. PCR-results per sea site for *D. lepeophtherii*, salmonid pox virus, *N.perurans* and *Ca.*B.cysticola

Results:

- 1059 fish, parallel qPCR results, histological and gross gill scores
- Additional 80 fish with parallel histology scores and qPCR results
- 50 fish with gross scores and qPCR results
- Majority of fish healthy
- Majority of gross and histology gill scores were low (Fig. 2 and 3)
- Minority of fish positive for salmonid pox virus and *N.perurans,* most positive for *D. lepeophtherii*, and *Ca.*B.cysticola (Tab.1.)
- Correlation between mean gross scores and histology total scores was low (correlation coefficient 0,29)
- Correlation between mean gross scores and qPCR ct-levels were low, from < 0.1 to 0.13
- Correlation between histology scores and pathogens observed and PCR-results mostly low (< 0.2)
- Moderate correlation for histology observation of amoeba and PCR-positivity of *N.perurans* (0.31) and observation of epitheliocystis and PCR-positivity for *Ca.*B.cysticola (0.33)

Conclusion: Preliminary results shows poor correlation between gross and histology gill scoring systems. In addition correlation between gross scores and qPCR ct-levels and between histology scores and qPCR ct-levels are poor. However, the lack of moderate to severe gill pathology and clinical gill disease in the fish limits our ability to make firm conclusions.

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APPENDIX 7

Prevalence and temporal development of salmon gill poxvirus infection in Atlantic salmon (*Salmo salar* L.) from freshwater to seawater

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Introduction: Salmon gill poxvirus (SGPV) was first detected in Norwegian farmed Atlantic salmon in 1995 and was characterized in 2015. The virus can cause outbreaks of clinical gill disease and high mortality in freshwater, but SPGV-infections have also been detected in the seawater phase of salmon farming. The aim of this study was to examine the temporal development of SGPV-infection during the freshwater phase and the first year after sea transfer in cohorts originating from sites with different freshwater systems and in autumn-transferred (S0) and spring-transferred (S1) Atlantic salmon smolts.

Materials and Methods:

- Eight cohorts (4 S0, 4 S1) of Atlantic salmon (A-H)
- From 2 flow through system (FT) and 2 recirculating system (RAS) freshwater sites
- Fish groups were transferred to 16 pens at 8 sea sites (2 pens/site)
- Gill tissue was collected for RT-qPCR-analysis:
  - 0-3 time points in freshwater, sampling 1-3
  - Every 4th to 6th week after sea transfer

Results:

- Prevalence ranged from 27 to 53% in the infected freshwater groups
- SGPV-infection was confirmed in 2 freshwater sites (1 RAS, 1 FT), in 4 fish cohorts (2 S0, 2 S1), at 6 time points
- Results are summarized in Table 1.
- Only one of the freshwater sites recorded mortality associated with SGPV-infection (site 4, group D)
- All sea sites tested positive for SGPV within the first 4 months after sea transfer
- Prevalence ranged from 0-100% across seawater sites, pens and time points

Conclusions:

All fish groups tested positive for SGPV at least once after sea transfer, independent of detection during the freshwater phase. This suggests infection occurs both in freshwater and seawater.

No clear effect of smolt type (S0 or S1) or freshwater production system on prevalence of SGPV during the seawater phase was observed.

Infections with SGPV appears to have a seasonal distribution in seawater, with higher prevalence of positive samples in late summer and autumn at most sites.

FW site System	Smolt	Fish group	Sampling 1	2	3	4	5	6	7	8	9	10	11	12	13
Site 1 FT	S0	A - A1	0 0/29	0 0/30	ND	95% 19/20	5% 1/20	0 0/20	0 0/30	0 0/30	0 0/30	43% 13/30	0 0/30	13% 4/30	7% 2/30
		A - A2				ND	0 0/20	0 0/20	0 0/30	0 0/30	0 0/30	10% 3/30	0 0/30	0 0/30	23% 7/30
	S1	E - E1	0 0/20	0 0/59	0 0/30	0 0/30	83% 25/30	80% 24/30	0 0/30	0 0/27	0 0/30	0 0/30	ND	ND	ND
		E - E2				0 0/30	40% 12/30	100% 30/30	0 0/30	0 0/29	0 0/30	ND	ND	ND	ND
Site 2 RAS	S0	B - B1	ND	ND	ND	90% 18/20	10% 2/20	5% 1/20	0 0/30	0 0/30	0 0/30	3% 1/30	37% 11/30	40% 12/30	0 0/29
		B - B2				100% 20/20	0 0/20	0 0/20	0 0/30	0 0/30	0 0/30	0 0/30	77% 23/30	33% 10/30	0 0/30
	S1	F - F1	0 0/30	0 0/30	ND	0 0/30	100% 30/30	27% 8/30	0 0/25	0 0/28	0 0/30	38% 10/26	3% 1/30	ND	ND
		F - F2				0 0/30	100% 30/30	43% 13/30	0 0/20	0 0/26	0 0/29	42% 8/19	0 0/29	ND	ND
Site 3 RAS	S0	C - C1	ND	ND*	ND	0 0/10	0 0/20	0 0/20	0 0/30	0 0/30	0 0/30	0 0/30	0 0/30	0 0/30	47% 14/30
		C - C2				0 0/10	5% 1/20	0 0/20	0 0/30	0 0/30	0 0/30	0 0/30	0 0/30	0 0/30	7% 2/30
	S1	G - G1	30% 9/30	0 0/30	ND	0 0/30	0 0/30	76% 22/29	13% 4/30	3% 1/30	57% 17/30	57% 17/30	0 0/30	ND	ND
		G - G2				0 0/30	0 0/30	33% 10/30	87% 26/30	3% 1/30	83% 25/30	0 0/30	0 0/30	ND	ND
Site 4 FT	S0	D - D1	47% 14/30	53% 16/30	ND	15% 3/20	0 0/19	15% 3/20	0 0/30	0 0/30	0 0/30	0 0/30	23% 7/30	3% 1/29	0 0/29
		D - D2				ND	5% 1/20	0 0/20	0 0/30	0 0/30	0 0/30	0 0/30	13% 4/30	3% 1/30	0 0/30
	S1	H - H1	27% 8/30	30% 9/30	ND	0 0/30	0 0/30	3% 1/30	0 0/30	13% 4/30	3% 1/30	3% 1/30	0 0/30	53% 16/30	ND
		H - H2				3% 1/30	0 0/30	10% 3/30	3% 1/30	10% 3/30	0 0/30	0 0/30	7% 2/30	23% 7/30	ND

Table 1. Overview of percent and number of salmon gill poxvirus positive fish per fish group and sampling point. S1 and S0 fish groups from the same freshwater sites are grouped together. Green squares indicate sampling during freshwater phase, blue during seawater phase, red SGPV-related mortality at site, dark are samplings where 1 or more fish were positive. *indicate that routine diagnostic sampling were positive for SGPV. ND = not done.

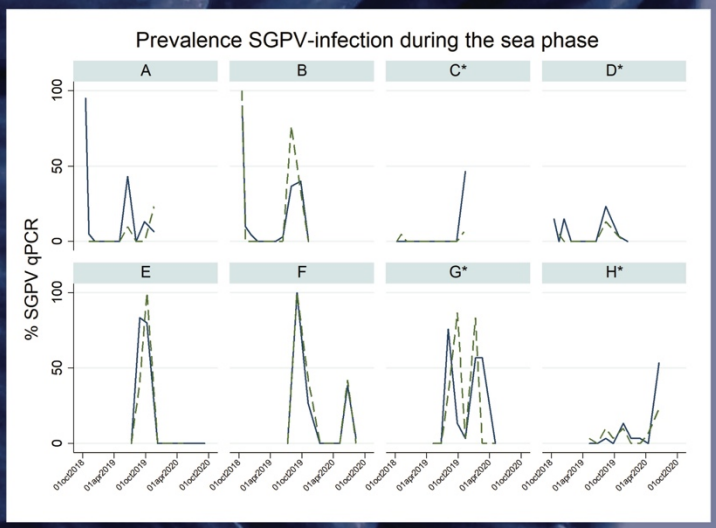

Figure 1. Overview of percent of salmon gill poxvirus positive fish per fish group in seawater. Solid line group 1, dashed line group 2. *indicate groups that tested positive for SGPV in the freshwater phase.

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