Norwegian University of Life Sciences
Faculty of Veterinary Medicine
Department of Production Animal Clinical Sciences

Philosophiae Doctor (PhD)
Thesis 2023:4

Monitoring fish health – a vital part of salmon farming management

Helseovervåking i lakseoppdrett – en viktig del av helsestyringen

David Weman Persson

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– a vital part of salmon farming management

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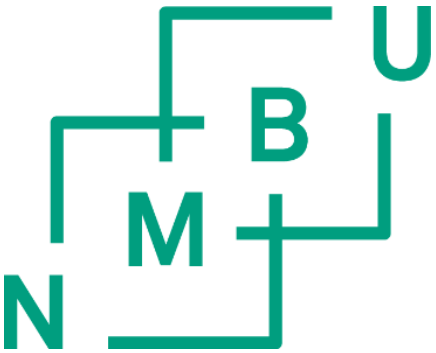
Ås (2023)



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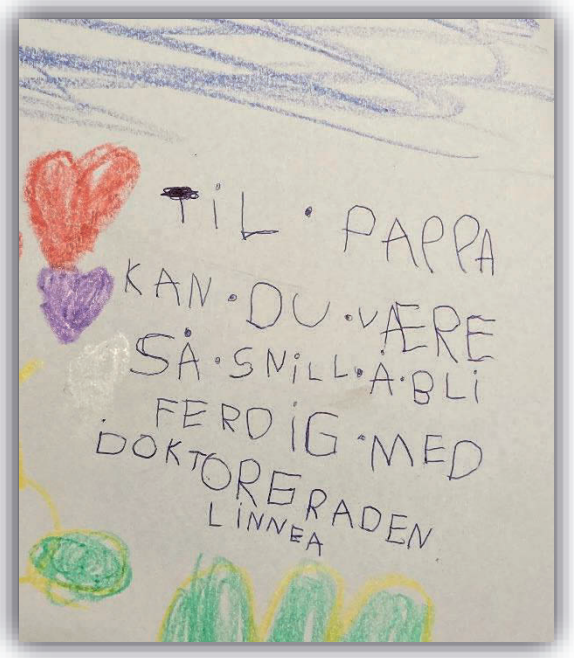
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scientific articles. Two of the studies relied on collaboration with SINTEF Ocean and project leader of the "MonMic"- project, Stine Wiborg Dahle. In addition to Stine, Deni Ribicic and Roman Netzer at SINTEF Ocean extensively contributed to the microbiome study through sample analyses and discussions. Finally, I could not have finished that paper without Stanislav Iakhno's assistance. Thank you all! In connection with the work on mortality causes, I am indebted to Paul Midtlyng, Sturla Romstad and Olav Jamtøy for providing invaluable input from their long careers working with health in salmon farming.

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Hvitsten, October 2022
David Persson

Table of Contents

1 Abbreviations and definitions

Alpha diversity	Variation of microbes in a single sample, assessed by species richness and diversity
Beta diversity	Variation of microbial communities between samples, assessed by the difference in taxonomic abundance profiles from different samples
Causality	The relationship between cause and effects
CMS	Cardiomyopathy syndrome
Disease	Partial or finite abnormality of structure or function with an identifiable pathological or clinicopathological basis, often with a recognizable syndrome or constellation of clinical signs.
Fish-group	Group of fish transferred to the same cage at sea
FT-farm	Flow through farm
ICD	International classification of diseases
ISA	Infectious salmon anaemia
IPN	Infectious pancreatic necrosis
IQR	Interquartile range
PD	Pancreas disease
Presmolt	Life stage of salmon before smoltification
Prevention, primary	Prevent disease before it ever occurs, monitoring risk factors and pathogens
Prevention, primordial	Prevent risk factors of diseases
Prevention, secondary	Early detection of disease to avoid spread
Prevention, tertiary	Reduce impact of a disease through treatment
RAS	Recirculating aquaculture system
S0	Smolt transferred to sea the same calendar year as start of feeding, typical transferred to sea in the autumn (before 1 year old)
S1	Smolt transferred to sea the calendar year following start of feeding, typical transferred to sea in the spring (fish 1 year old)
Salutogenesis	Theory of health, a concept studying factors contributing to good health

Shannon index	Alpha diversity metric of species richness and abundance, measuring both the number of species and the inequality between species abundances.
Sources of variation	Statistical term, identifying the amount of variation to each random effect (or level) in a regression model
Stocking period	When in year the fish was stocked (typically spring or autumn)
WHO	World Health Organisation
Year class	Salmon transferred to sea in the same calendar year

2 List of papers

Paper I

Analysing mortality patterns in salmon farming using daily cage registrations

Authors: David Persson, Ane Nødtvedt, Arnfinn Aunsmo, Marit Stormoen

Published: Journal of Fish Diseases 2021, DOI: 10.1111/jfd.13560

Paper II

Real-time monitoring of cause-specific mortality and losses in industrial salmon farming

Authors: Arnfinn Aunsmo, David Persson, Marit Stormoen, Sturla Romstad, Olav Jamtøy, Paul Midtlyng

Accepted, 18th of October 2022 in Aquaculture

Paper III

Variations in mucous cell numbers in gills of Atlantic salmon (*Salmo salar*) presmolt in commercial freshwater farms in Norway

Authors: David Persson*, Håvard Bjørgen*, Alexander Figenschou, Linn-Anett Hillestad, Erling Olaf Koppang, Ane Nødtvedt, Marit Stormoen

Published: Journal of Fish Diseases 2020, DOI: 10.1111/jfd.13263

Paper IV

Bacterial community composition in gill mucus and corresponding environment in four commercial Atlantic salmon RAS

Authors: David Persson, Stanislav Iakhno, Stine Wiborg Dahle, Roman Netzer, Renate Sandberg, Henning Sørum, Deni Ribicic

Manuscript

*Shared first authorship

3 Abstract

Mortality represents a major challenge in the salmon farming industry. In Norway the mortality in the marine phase of the salmon production cycle has been 15-20% annually the recent years. This situation is not sustainable for the fish, the farmers or society. To prevent mortality throughout the production cycle, fish-groups at risk of deteriorating health must be identified early, regarding both disease development and stage in the production cycle. Hence, robust population health monitoring methods are needed, which will provide farmers with information to make knowledge-based decisions to improve fish health.

The aim of the thesis was to investigate monitoring methods that can be used to improve fish health management in salmon farming. This was approached through four studies investigating mortality patterns, cause-specific mortality classification, gill mucous cell count and gill mucus microbial composition, respectively, as potential factors relevant to monitoring fish health status in salmon farming.

Mortality patterns in salmon farming were investigated through a retrospective study of fish-groups from two commercial fish farming companies, describing the cause-specific mortality during the production cycle and investigating sources of variation in mortality between the hatchery, marine farm, and the fish-group. Most of the variation in mortality was attributed to the fish-group. Based on the information from the cause-specific mortality registrations, smolt-related mortality was found to be the major cause of death during the first six months of production, while handling and treatment was the overall dominating cause of death in the full production cycle. However, this varied extensively between the fish-groups.

The second study further explored the system of cause-specific mortality and suggested a unique classification code. A system for classifying causes of mortality based on underlying cause was created for salmon farming inspired by the human mortality classification system. The proposed standard has a three-level hierarchical structure of mortality causes. This accommodates different levels of details when mortality causes are registered and enhance the information possible to retrieve from the system.

To target early responses of the immune system, variation in gill mucous cell count was studied as a potential method of monitoring health status. Salmon presmolt from six commercial hatcheries was sampled and mucous cells were histologically quantified based on certain criteria. The counts varied among both fish and farms. When "farm" was included as an independent variable in a regression model the proportion of variation in mucous cell counts explained by the model was twice as high compared to when only fish size was included. This indicates that the variation depends on farm-related factors.

A subset of the fish (from four recirculating aquaculture system (RAS) facilities) included in the study of mucous cells was also sampled for gill mucus for microbial composition analyses using 16S rRNA gene sequencing. Quantification of extracted bacterial DNA in the gill mucus samples showed low levels in general, but fish from one farm had considerably more bacterial DNA compared to the others. Samples from the same farm were also compositionally different based on beta diversity metrics, compared to the others as one group. Assessed by the Shannon index as an outcome in a regression model, sources of variation attributed most variation to the individual fish, suggesting gill microbial structure was linked to the individual fish. No associations between gill microbial diversity and specific production parameters were detected.

In conclusion, cause-specific mortality classification was found to identify important causes of mortality in the production cycle, thus providing farmers with substantial health information with limited effort and at little cost. The tool can further be applied at desired level of detail and adapted to the needs of each farmer. Novel methods of gill mucous cell count and microbial composition identifies variation among farms and individual fish for the different measured parameters. This variation can potentially be exploited to detect early signs of reduced health in the fish. However, further studies are necessary to establish the causal association between these factors and fish health before they can be used in systematic monitoring of health in salmon farming.

4 Norsk sammendrag

Dødelighet i oppdrettsnæringen er en stor utfordring i dag. Norsk lakseoppdrett har de siste årene hatt mellom 15 og 20% dødelighet i sjøfasen. Dette er ikke bærekraftig, hverken for fisken, oppdretterne eller samfunnet. For å forebygge dødelighet gjennom produksjonssyklusen må fiskegrupper som har økt risiko for dårligere helse identifiseres tidlig. Derfor er robuste overvåkingsmetoder på populasjonsnivå nødvendige, det vil gi oppdretterne nyttig informasjon for å kunne ta kunnskapsbaserte avgjørelser i arbeidet med å forbedre helsesituasjonen til fisken.

Målet med doktorgraden var å undersøke overvåkingsmetoder som kan brukes til å forbedre helsestyringen i oppdrettsnæringen. Dette ble gjort ved å undersøke mulige faktorer som kan gi informasjon om helsestatus, henholdsvis dødelighetsmønster, årsaks-spesifikk dødelighetsklassifisering, slimceller på gjellene og mikrobiell sammensetting på gjellenes slimlag.

Dødelighetsmønster i lakseoppdrett ble studert retrospektivt med utgangspunkt i den årsaks-spesifikke dødeligheten gjennom produksjonssyklusen i fiskegrupper fra to oppdrettere. I tillegg ble varianskomponenten i dødelighet undersøkt mellom settefiskanlegg, sjøanlegg og fiskegrupper. Fra informasjonen om årsaks-spesifikk dødelighet i produksjonsdataene ble smolt-relatert dødelighet identifisert som den største dødsårsaken de første seks månedene etter sjøsetting. Totalt i hele produksjonssyklusen var det håndtering og behandlingsdødelighet som dominerte. Samtidig var det stor variasjon mellom fiskegruppene, både i antallet døde og hva som var dominerende årsak. Betydningen av fiskegruppen som enhet ble også støttet av varianskomponent-analysen hvor hovedandelen av variasjonen i dødelighet ble knyttet til fiskegruppen.

I den andre studien ble klassifisering av dødsårsaker ytterliggere studert. Studien resulterte i et forslag til en standardisert liste over dødsårsaker. Systemet ble basert på samme tankegang som den humane dødsårsaksregistreringen, hvor den underliggende dødsårsaken er utgangspunkt ved registrering. Videre er de forskjellige dødsårsakene gruppert på tre nivåer, med økende detaljgrad nedover i systemet. Det gjør systemet fleksibelt, både for bruk på merdkanten ved registrering

av årsakene, men også når informasjonen skal brukes videre i forebyggende helsearbeid.

For å studere tidlig indikasjon på sykdom, ble variasjon i antall slimceller på gjellene undersøkt som et mulig mål på fiskens immunrespons. Prøver av gjeller fra seks kommersielle settefiskanlegg ble undersøkt histologisk, og antallet slimceller ble telt ut fra spesifikke kriterier. Resultatene viste at antall slimceller varierte betraktelig både mellom anlegg og fiskeindivider. Settefiskanlegg hadde mye å si for forklaringsgraden (R2) når det gjelder prediksjon av slimceller på gjeller. Forklaringsgraden ble dobbelt så stor i en regresjonsmodell med både «settefiskanlegg» og «fiskestørrelse» inkludert sammenlignet med en modell kun med «fiskestørrelse» som forklaringsvariabel.

I den fjerde studien ble slimlaget på gjellene analysert med 16S rRNA gen-sekvensering for å studere sammensetning av bakterie-mikrobiomet. Dette ble gjort på et utvalg fisk (fra anleggene med resirkuleringsteknologi (RAS)) som var inkludert i studien om slimceller. Kvantifisering av bakterielt DNA indikerte generelt lave nivåer av bakterier i slimlaget på gjellene, men et anlegg skilte seg ut med høyere nivåer. Basert på beta diversitet delte fisken seg i to distinkte grupper, der fisk fra det samme anlegget skilte seg ut med uttalt forskjellig bakteriesammensetning sammenlignet med de andre. Vurdert med Shannon indeks som utfall i en regresjonsmodell, knyttet varianskomponentanalysen størst andel av variasjonen til den individuelle fisken. Det ble ikke funnet noen sammenheng mellom uttrykk i gjellens bakterie-mikrobiom og undersøkte produksjonsparameterer.

Avslutningsvis, med data fra årsaks-spesifikk dødelighetsregistrering kan man identifisere relevante dødsårsaker i produksjonssyklusen. Derfor gir dette verktøyet vesentlig informasjon om fiskepopulasjonen sin helsestatus. Det er dessuten enkelt for oppdretteren å bruke. De nyere metodene som er undersøkt (antall slimceller på gjellene og mikrobiom i slimlaget) viser en variasjon mellom anlegg og fiskeindivider i de målte parameterne. Denne variasjonen kan potensielt utnyttes for å detektere tidlige tegn på dårlig helse hos fisken. Mer forskning på sammenhengen mellom disse faktorene og fiskens helse er nødvendig før metodene kan tas i bruk som systematisk overvåkingsverktøy i oppdrettsanlegg

5 Synopsis

5.1 Introduction

Salmon farming is an intensive system of animal production in which each farm has several hundred thousand or even millions of individuals in one population. The large number of individuals living together in a confined space makes health management vital. If health deteriorates, disease and mortality rapidly become a challenge in production and, consequently, fish welfare and economic profit diminishes quickly. Disease prevention has been a mainstay of fish farming since production was industrialised (Gudding & Van Muiswinkel, 2013; Pettersen, Osmundsen, Aunsmo, Mardones, & Rich, 2015; Shepherd & Poupard, 1975). However, how to maintain health in fish populations – and not only avoid disease – has not been studied extensively in veterinary medicine. In human medicine, we find the term "population health monitoring" which is defined as "the regular and institutionalised production and dissemination of information and knowledge about the health status of a population and its determinants, aimed at informing policy-making." (Verschuuren & van Oers, 2019). Even though a comparison between human and salmon health might seem peculiar, there are similarities when considering population-based concepts that could be applicable across species. Population health monitoring, as defined above, implies that knowledge about health is based on information which originates from registered and collected data describing the health status of a population. In human health, this knowledge is used in decision-making to improve health policy in society. Knowledge-driven decision-making is also vital in population-based production animal husbandry, for example in dairy farming (Østerås et al., 2007). Population health monitoring would therefore be highly relevant to the aquaculture industry. Identifying factors that improve fish health, and not only prevent diseases, would preserve health at an earlier stage and increase survival in a population. This thesis will explore different methods to monitor fish health status which can provide the farmer with information to make knowledge-based decisions in how to improve the production as illustrated in Figure 1.

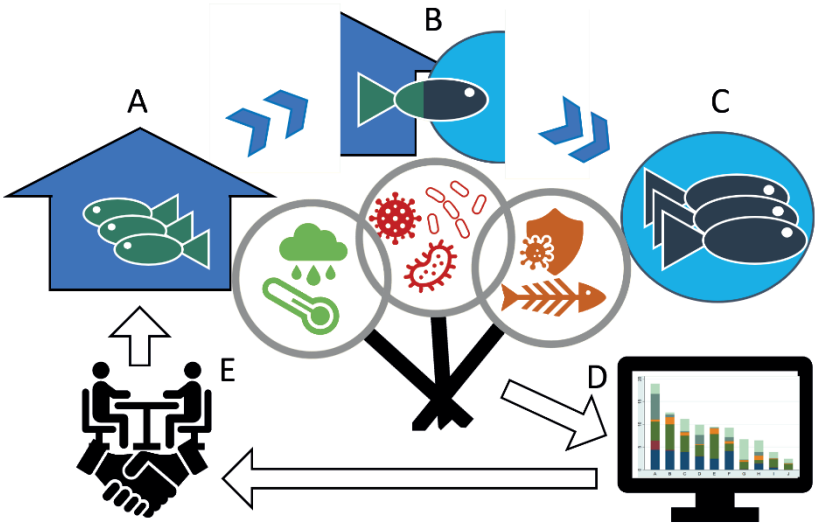

Figure 1. Illustration of different processes included in the cycle of fish health management in salmon farming. Monitoring (magnifying glasses) of factors related to fish health (green: environmental factors, red: pathogens, orange: host, here illustrated by mortality and immune status) in the production (A-C) makes up data (D) which provide the farm-management with information to make knowledge-based decisions (E) in how to improve the production (A-C) and subsequently fish health. A: Land-based production from egg to smolt, B: smoltification and transfer to sea, C: Marine phase of the production.

5.1.1 Farming of salmon

Norwegian salmon farming is a hierarchical production system in which four breeding companies provide eggs to around 200 hatcheries, these produce the salmon smolt transferred to one or more of approximately 800 farms at sea (Figure 2) (Directorate of fisheries, 2022a, 2022b; Gåsnes et al., 2021; Næve, Korsvoll, Santi, Medina, & Aunsmo, 2022). Salmon is an anadromous fish, living the first part of the production cycle in hatcheries using freshwater (Figure 1A) and is adapted to a life in seawater after smoltification. Smoltification is the physiological preparation to tolerate the transition (Figure 1B), both physiological and spatial, from the freshwater environment in the hatchery to the grow-out phase in marine farms (Figure 1C). This mirrors the life of wild salmon, which hatch and spend their early life in fresh-water rivers before migrating into the sea when they grow older.

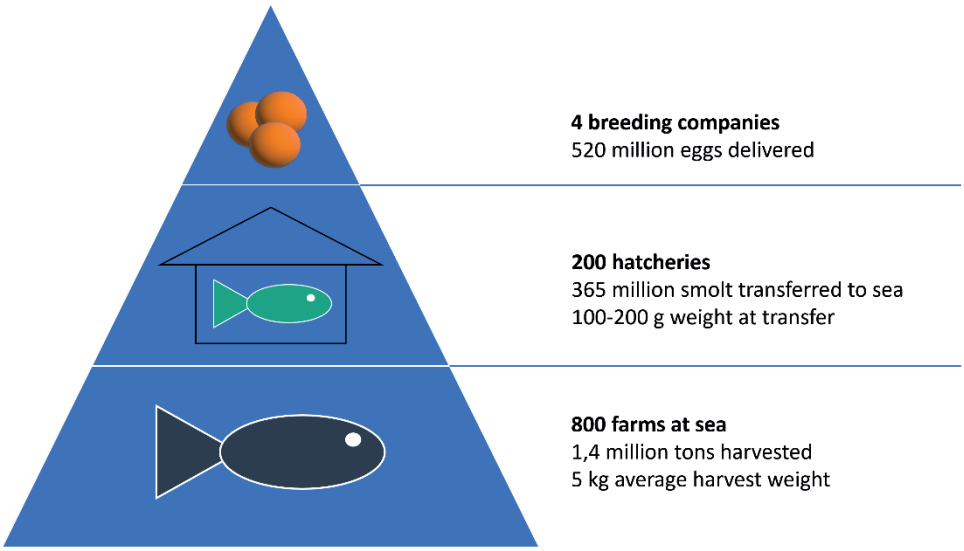

Figure 2. Illustration of the hierarchy in Norwegian salmon production with numbers indicating annual production at each level. Based on numbers from the Directorate of Fisheries for 2020 and Næve et al. (2022).

Breeding and genetics

Four companies (Aquagen, Benchmark Genetics, SalMar and Mowi) currently produce eggs for the Norwegian salmon industry (Rosendal & Olesen, 2022). Aquagen and Benchmark Genetics work exclusively with genetic improvement, breeding and sell eggs, whereas SalMar and Mowi are primarily farming companies breeding for their own production (Rosendal & Olesen, 2022). All four companies work extensively with selective breeding and genetic improvements of their genetic stock. Selective breeding of salmon started 50 years ago and at least eleven generations have been bred since that time (Næve et al., 2022). The traits selected for in the breeding programme vary across time and companies. Exemplified by Aquagen's breeding programme, selective breeding traits can be grouped into three major feature groups: growth, health and quality. Growth is the only trait included from the first generation, whereas health and quality have been added in later generations, with different weighting of traits between generations (Næve et al., 2022). At Aquagen, for example, selective breeding has reduced the production time at sea (to reach 4 kg) by seven months between generation 7 and 13 (Næve et al., 2022). In terms of fish health, one of the most remarkable success stories in the history of aquaculture is said to be the selective breeding of increased resistance to the viral disease infectious pancreatic necrosis (IPN) using genetic markers

(Hillestad, Johannessen, Melingen, & Moghadam, 2021; Moen, Baranski, Sonesson, & Kjøglum, 2009). After inclusion of the IPN resistance marker in the breeding programme, the number of IPN outbreaks in Norway declined from 223 reported cases in 2009 to 20 in 2021 (Sommerset et al., 2022). Selective breeding and the continuous genetic improvement of the fish produced is a cornerstone of fish farming and will be important for the future growth of the industry and vital in long-term improvements in fish health.

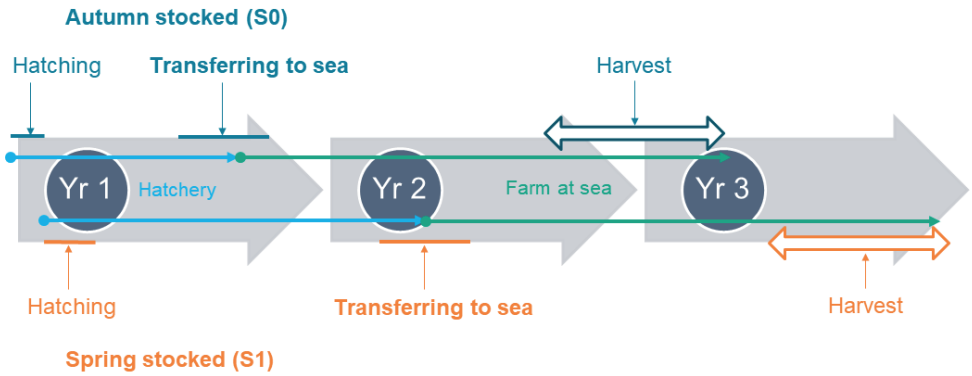

Figure 3. Illustration of the production timeline in salmon farming, differentiating between fish stocked in autumn (dark blue) and in spring (orange). Indicating events of hatching, transfer to sea and harvest. Light blue arrows show the production time in freshwater in the hatcheries and green arrows show the production at sea.

Land-based production

Traditionally, salmon hatcheries have been built near a freshwater source (river or lake) re-routing part of the water stream through the farm, a technology known as flow-through (FT). However, limitations in water volume accessible from these rivers and lakes ultimately limits the number of smolts produced. In addition, seasonality affects several water parameters unpredictably. The above-mentioned factors create a constraint on the production capacity necessary for the continuous delivery of more and larger fish (Dalsgaard et al., 2013). One way to overcome this is using another water treatment technology called a recirculating aquaculture system (RAS).

RAS

The water in a RAS is recirculated in a closed rearing environment within the farm. The fish use oxygen and excrete metabolic waste products, meaning a closed system needs to replace the oxygen and remove waste products to ensure suitable environment for the fish. In a traditional FT-system, the constant flow of water through the facility caters for several of these needs with reduced management to sustain the living conditions compared to a RAS where this task is vital.

Following the water from the outlet of the fish tank in a RAS, the water is cleaned of waste products and particles through an integrated system of water treatment procedures before returning to the fish tank to complete the recirculation loop. The water treatment includes mechanical and biological filters to remove particles and nitrogen compounds from the water before the water is stripped from CO_2 and aerated (van Rijn, 2013). It is critical to control the amount of ammonia (NH^3-N and NH^{4+}-N) in a RAS as it can accumulate and become toxic if the bacterial nitrification is insufficient (Davidson, Good, Williams, & Summerfelt, 2017). To maintain a stable environment in a RAS it is also important to control the level of organic particles derived from faeces, excess feed and biofilm. The largest particles are removed by mechanical filtering of the water, but the smaller ones pose more of a challenge when it comes to control (Chen, Timmons, Aneshansley, & Bisogni, 1993; Fernandes, Pedersen, & Pedersen, 2017). Particles in the water can cause damages to the gills and induce a general stress response (Awata, Tsuruta, Yada, & Iguchi, 2011; Becke, Schumann, Steinhagen, Geist, & Brinker, 2018; Bruton, 1985), or indirectly pose a threat to fish health by reducing the effect of filtering (de Jesus Gregersen, Pedersen, Pedersen, & Dalsgaard, 2019). Therefore, to ensure stable and good water quality in RAS, close monitoring of a wide range of water parameters (e.g., ammonia, hydrogen sulfide, CO_2, temperature pH, turbidity) is important to ensure good fish health and welfare (Sommerset et al., 2022).

Recirculation of close to 100% of the water in farms with RAS have solved the challenge of water availability which has been a constraint to production in traditional FT-farms. In addition, water parameters are not affected by seasonality and the water temperatures are stable all year round, allowing for a more rapid and stable growth of fish (Dalsgaard et al., 2013). The expansion of hatcheries built to accommodate increasingly bigger sizes of fish in Norway is evident in the number of fish exceeding 250 grams when transferred to sea. This number has increased from

about 3% of the fish transferred to sea in 2018 to more than 15% in 2020 (Directorate of Fisheries, 2022b).

Production at sea

In traditional commercial fish farming, fish spend between seven and 12 months in the hatchery and 12 to 18 months in the sea (Figure 3). The fish are transferred to sea at a weight of 100 to 200 grams (average weight 2016-2019: 131 grams) and the weight at harvest is around five kg (Næve et al., 2022). Traditionally, salmon are stocked in either the spring (S1) or the autumn (S0), where "S1" refers to fish being 1 year old when transferred to sea while "S0" implies that fish have spent less than a year at the hatchery. However, due to developments in hatchery technology, fish nowadays can be delivered to sea all year round and at different weights. At sea, the farms are managed by the principle of "all-in, all-out", allowing only one age group in the farm at a time. In practice, since production time in sea is between 12-18 months (Figure 3), fish (either S0 or S1) will be stocked bi-annually at each farm.

5.1.2 Fish health in salmon farming

5.1.2.1 Definition of health

Health is surprisingly difficult to define. Gunnarsson (2006) performed a thorough review of 500 veterinary textbooks to investigate the definition of animal health. He found that definitions of health (and disease) were disperse and poorly investigated, considering the fundamental meaning "health" and "disease" have in veterinary medicine (Gunnarsson, 2006). However, the study suggested that a definition of animal health could be divided into five themes or concepts:

1. Normality
2. Biological function
3. Homeostasis
4. Physical and psychological well-being
5. Productivity

The study did not settle on one definition and acknowledged the fact that different disciplines traditionally have different definitions of health. For example, clinical examination mainly relies on the concept of "normality" when assessing the health of an animal. Including blood samples or other analytic tools in a health assessment would probably bring us closer to a definition of health based on biological function or homeostasis, as pathology and pathophysiology investigations help us interpret how well the animal is coping with its surroundings. If we include physical and

psychological well-being, we approach the definition of human health. The World Health Organisation (WHO) defines human health as "a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity" (World Health Organization, 2022). In veterinary medicine, the concepts of health and welfare are closely related. However, there might be a challenge to include welfare in a definition of health. An unhealthy animal would commonly be considered to have reduced welfare, but an animal in good health can also have reduced welfare (Gunnarsson, 2006; Segner et al., 2012). Therefore, health would more accurately be considered an important part of welfare rather than the other way around. The fifth approach to health, productivity, is uncommon as the sole definition of health according to Gunnarsson (2006). However, productivity is an important factor when assessing the health of a production animal, which is unique to veterinary medicine. The other four concepts could be regarded as universal, applicable to all animals and humans (Gunnarsson, 2006). Health in aquaculture has traditionally been interpreted as the absence of disease rather than through a positive definition (Foyle, Hess, Powell, & Herbert, 2020; Segner et al., 2012). However, Segner et al., (2012) argue that health should be defined as "the ability of an animal to perform normal physiological functions and to maintain homeostasis" (Segner et al., 2012). This correlates to the second and third definitions of health given by Gunnarsson (Gunnarsson, 2006).

5.1.2.2 Fish welfare

Human society has opinions about how animals should be treated, and these opinions are often based on the concept of welfare. Expectations regarding welfare in production are ultimately transferred into legislation. The Norwegian Animal Welfare Act protects all vertebrates, decapods and honey bees in human custody (Dyrevelferdsloven, 2009), clearly showcasing the importance of welfare as a prerequisite in animal husbandry. However, the legislation also suffers from uncertainty as to how to define welfare. To be efficient in a regulatory perspective, acceptable (legal) welfare must be distinguished from unacceptably low (illegal) levels of welfare. In order to know what constitutes "good" and "bad" welfare, objective and reproducible measurements of welfare are needed for transparent and equal law enforcement.

The concepts of health and welfare are interwoven with welfare understood as more overarching than health (Segner et al., 2012). Animal welfare, as it applies in aquaculture, has been defined by the extensive "Fishwell" project

(.fhf.no/prosjekter/prosjektbasen/901157/) as “the quality of life as perceived by the animal itself” (Noble et al., 2018). Segner et al. (2012) further describe welfare based on the “five freedoms”, a concept in animal welfare established in the 1960s by the Brambell Committee (Brambell, 1965). The authors compress the list into three categories: feelings, nature and function (Segner et al., 2012). “Feelings” in this sense refers to the sentient animal and how to reduce its experience of pain and fear. “Nature” focuses on the ability to express natural behaviour, while “function” is about coping with the environment, maintaining homeostasis and normal biological functions. It is challenging to know how to approach an animal’s experience of its circumstances since we do not know how to measure its cognitive expressions or capabilities. Human welfare, by comparison, is easier to establish in this context as it can be assessed verbally. Thus, animal welfare would be assessed mainly based on “function” and “nature”, with the aim of this also being positive for the third category, “feelings”. Interestingly, “nature” and “function” also resemble Gunnarsson’s second (biological function) and third (homeostasis) definitions of health (2006). Thus, any progress in monitoring health through biological function and homeostasis is also valuable when assessing animal welfare.

5.1.2.3 Health challenges in production

Several studies have identified the period following sea transfer as having the highest risk of mortality during production (Bang Jensen, Qviller, & Toft, 2020; Salama, Murray, Christie, & Wallace, 2016; Soares, Green, Turnbull, Crumlish, & Murray, 2011). In the last phase of the freshwater stage in the hatchery, the salmon undergo a major physiological process called smoltification. Smoltification is the transition between life stages, going from fresh water to a life in salt water. It is an energy-demanding physiological process that temporarily affects the immune system negatively (Johansson, Timmerhaus, Afanasyev, Jørgensen, & Krasnov, 2016; McCormick, 2012). Following smoltification in salmon production, the fish change environment (from fresh- to salt water) when they are transferred from the hatchery to the sea. The transport between these locations is in itself stressful for the fish. Thus, a series of events with potential effects on fish health take place in a short time period following smoltification. For the farmers, the key to success is controlling the smoltification process and ensuring correct timing of the fish transfer. This includes controlling the environment (e.g., light, feeding, salinity), size within the group of fish, and monitoring the smoltification status continuously until transfer (Iversen et al., 2005; Johansson et al., 2016; Poppe & Bergh, 1999). Good health status is vital to ensure simultaneous smoltification in a group of fish. If

smoltification fails, or becomes insufficient in a fish-group, there is an increased risk of salmon displaying reduced growth (stunting) and of disease development in the population (Striberny et al., 2021). Thus, monitoring health status in the last part of the production cycle in the hatchery could potentially benefit health and productivity, both in the short and long term.

In a hatchery, there are several means to prevent the introduction of pathogens and reduce the risk of disease spread between tanks of fish within a farm. Important biosecurity measures include disinfection of intake water and sectioning the farm into suitable compartments to avoid spread of disease throughout the entire hatchery (Ervik et al., 2020). These measures of protection are practically impossible in farms operating in the open sea. The water environment surrounding the cages is a factor that is difficult to control. As a result, pathogens in the sea may spread between cages and between farms. In the 1980s and 90s, Norwegian salmon production suffered from widespread disease burden deriving from bacterial and viral diseases such as furunculosis (*Aermonas salmonicida* subspecies *salmonicida*), cold water vibriosis (*Vibrio salmonicida*) and infectious salmon anaemia (ISA virus) in farms at sea. One of the biosecurity measures taken to combat these disease challenges was the implementation of the "all-in, all-out" principle, namely having only one age group at a farm location and a fallowing period before next stocking of fish. This effectively reduced the number of ISA outbreaks in the 1990s and helped reduce infection pressure and the spread of bacterial diseases (Ervik et al., 2020). However, the development of effective vaccines is considered the major contribution to the successful handling of bacterial diseases in marine salmon aquaculture (Gudding & Van Muiswinkel, 2013). Today all farmed salmon are vaccinated in the hatcheries, before smoltification, to increase the resistance against infectious diseases in the marine phase. Indisputably, the important preventive measures of regulated stocking periods and vaccination have been the foundation to the growth of salmon production seen up until today.

Nevertheless, there are still several disease challenges present, especially in the sea phase. Today, these are primarily represented by the viral diseases cardiomyopathy syndrome (CMS) and pancreas disease (PD) and the handling of the ectoparasite salmon lice (*Lepeophtheirus salmonis*) (Sommerset et al., 2021). Of those, salmon lice would be considered the major challenge for the industry in Norway. Salmon lice is also a dominant factor in the growth-stagnation of production seen the last 10 years (Iversen, Asche, Hermansen, & Nystøyl, 2020; Iversen et al., 2019). The total

economic cost of keeping salmon lice levels in the industry below the governmental limit increased from an estimated one billion NOK in 2011 to more than five billion NOK in 2018 (Iversen et al., 2019). The governmental regulation limits the acceptable salmon lice abundance in production to 0.5 adult lice per salmon individual (Forskrift om lakselusbekjempelse, 2013). Treatment operations combating lice infestation (delousing) is also a driver of salmon mortality in the industry and predispose the fish to other diseases. (Iversen et al., 2020; Sommerset et al., 2021). Widespread resistance to most pharmaceuticals available for controlling salmon lice has forced a shift in the industry towards the use of mechanical and thermal methods for delousing the fish (Figure 4). These methods include extensive handling of fish, which negatively impacts health and welfare and increases mortality (Overton et al., 2019; Sviland Walde, Bang Jensen, Pettersen, & Stormoen, 2021). For example, when treatments are performed on a well-boat, the individual salmon are moved from the net pen to the well-boat and back to the net pen again using pumps.

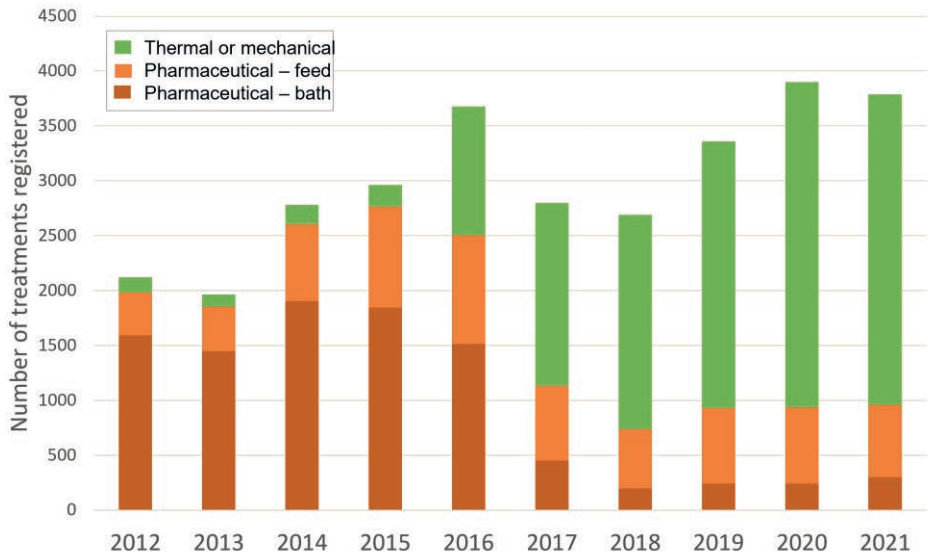

Figure 4. Graph illustrating the shift from pharmaceutical to thermal/mechanical treatment methods for sea lice in Norway between 2012 and 2021. Based on official records (reported weekly) retrieved from barentswatch.no.

Prevention at all levels is thus important to reduce salmon lice infestation and avoid delousing events and the associated handling of fish. Here, biosecurity concerns from the 90s (as described earlier) have been extended further, to allow only one stocking group (S0 or S1), within a year class, stocked in a geographical region. The regions and stocking organisation are specified by the farming companies. This is done to avoid having younger fish stocked in an area with high infection pressure (generated from older fish in the same region) and to make the effect of the fallowing period stronger when a larger region has to be fallowed simultaneously – not just the single farm (Ervik et al., 2020; Forskrift om lakselusbekjempelse, 2013). Different methods to shield caged fish from sea lice infestation have been developed to reduce the number of treatments needed at the farm level (Geitung et al., 2019; Stien, Lind, Oppedal, Wright, & Seternes, 2018). However, despite these preventive measures, the number of treatments reported in the Norwegian salmon population has never been higher than in the years 2020 and 2021 (Figure 4).

5.1.2.4 Improving health through preventive medicine

Health is not constant throughout life. An individual's health status will vary, and a reduced health status can be manifested by acute changes or a slow progression into failing health. In aquaculture, health is often affected by events in production (Aunsmo et al., 2008), like disease outbreak or bad weather, or linked to the factors in the production environment like technical failure of water treatment in land-based facilities for example. Traditionally, veterinary medicine is good at detecting and acting upon acute onset of known disease or disorders. More difficult are situations where health deteriorates slowly over time and the cause is ambiguous. However, in both circumstances, early detection and action are desirable to prevent further development.

In 1974, E.A. Clarke tried to envisage prevention in human medicine by the following statement: "If you ask healthy people what they want most, you will get as many answers as there are people. Ask a man when he is ill or has an inkling of premature death and you will get the truth. He wants more than anything else to be healthy" (Clarke, 1974). This shows the challenge with prevention. Most people will not think of their health until it is too late.

Specialty	Preventive medicine			"Acute" medicine	
Paradigm	Theory of health (Salutogenesis)		Theory of disease (Pathogenesis)		
Tools	Health promotion	Health protection	Disease prevention	Disease treatment	
Target level	Macro (national)	Meso (site and cage - fish-group)			Micro (individual)
Level of prevention	Primordial prevention	Primary prevention	Secondary prevention	Tertiary prevention	
Fish health in practice	Fish health strategy and management at farm		Clinical fish health work at the farm (fish health services)		

Figure 5. Concepts in aquatic veterinary preventive and acute medicine, described as a matrix with suggested boundaries. For further explanation, refer to the text. Inspired and adapted from the illustration for human medicine described in Jadotte et al. (2021).

To prevent disease and preserve health, knowledge about the transition from a healthy state to a diseased state is necessary, as is the investment of resources to find that knowledge and act upon it (Clarke, 1974; Jadotte & Lane, 2021). In light of the COVID-19 pandemic, Jadotte et al. (2021) have described preventive medicine in human health and suggest how it should evolve further. Tools developed within human preventive health might also be of interest for population-based parts of veterinary medicine, such as aquaculture. Jadotte et al. (2021) published an illustration of different concepts of health and disease and the relation between them in human medicine. In Figure 5 these concepts are adapted and modified for aquatic veterinary medicine. The theory of health, or salutogenesis, is the lesser-known counterpart to the theory of disease, or pathogenesis. Salutogenesis has to do with how individuals stay healthy, which overlaps with preventive medicine. The tools within the paradigms are health promotion and health protection in salutogenesis, and disease prevention and disease treatment in pathogenesis. Disease prevention and treatment are probably better-known concepts compared to health protection and health promotion. In human health, health promotion involves increasing people's control over their health and improving it across the whole population, not only among those at risk of developing disease (Jadotte & Lane, 2021). An equivalent example in salmon farming could be feed with health enhancing additives given to the entire population at a farm. Health protection is slightly more specific, targeting a situation where there is a known risk for deteriorating health. An example could be to stop feeding fish if oxygen levels in the

water's surface layer drop to reduce the risk of morbidity and mortality (since fish feed in the upper water column). Health protective measures could also be exercised at a national level, with the regulatory requirement of a fallowing period between stocking of fish to reduce infection pressure and protect the health of the fish. The target level refers to which type of population is involved. In fish production, the cage or tank would almost always be considered the lowest epidemiological unit ("meso" in Figure 5), except with brood-stock fish, which at certain points in life would be considered at an individual level ("micro"). As previously described, there is also a national focus in preventive aquatic medicine, which is reflected in the figure as the "macro" target level.

"Level of prevention" is an expression commonly used in epidemiology and the field of preventive medicine. It refers to different health prevention measures or, in adverse situations, how to recover from disease (Mardones, 2020). The first level of prevention in aquatic medicine is called primordial prevention according to Mardones (2020). This includes actions taken to avoid the emergence or development of risk factors (for disease or deteriorating health) in the first place. One example of an action in primordial prevention is choosing the spatial location of farms, which potentially affects the infectious pressure upon the farm depending on factors such as distance to other fish farming units and environmental conditions. Primary prevention consists of identifying (and preferably monitoring) risk factors with a causal relationship to a disease or a health challenge. Consequently, the disease or health challenge can be avoided at an early stage, before disease development. Secondary prevention includes vaccination. A tertiary response involves treating a disease and preventing further spread.

The last row in the figure describes how the different concepts are applied practically in salmon farming in Norway. Most preventive work is performed as part of a strategy of fish health management at farms, often planned ahead of a stocking period whereas acute events of disease outbreak and treatment are part of the clinical fish health work during the production cycle.

5.1.2.5 Fish health management

Fish health management can be explained as a management practice which is designed to prevent fish diseases (Francis-Floyd, 2005; Kibenge & Powell, 2020). The authors of the book *Aquaculture Health Management,* moreover, argue that health management in aquaculture is largely confused with managing disease

(Kibenge & Powell, 2020). This is similar to the argument described earlier that health is something more than just freedom from disease. In health management, the aim is to enhance the health of the population. Disease prevention is obviously important, but for farmers the focus should also be to keep the fish healthy throughout the production cycle, and therefore fish health should be more than simply absence of disease.

In the end, the number of fish that reach harvest weight and are slaughtered is the crude result of fish health management for the farmer. Biological losses, in terms of reduced growth and mortality, have large economic implications for the industry (Aunsmo, Valle, Sandberg, Midtlyng, & Bruheim, 2010; Iversen et al., 2020; Pettersen, Rich, Jensen, & Aunsmo, 2015). Increased survival of fish in production has an immediate positive effect on revenue. This can be demonstrated with an example where 1 000 000 salmon are transferred to a sea farm. Mortality during production is 15% resulting in 850 000 fish reaching harvest at 5 kg. Assuming a sales price of 50 NOK/kg, this will equal 212.5 million NOK. Due to improvements in fish health management the next stocking period, mortality is reduced to 5% meaning 950 000 fish reach harvest for a revenue of 237.5 million NOK, a 12% increase compared to the previous stocking period. Further, the cost per kg of salmon produced is reduced since the biomass by which the cost is divided upon increased. For simplicity, the example assumes equal cost of production, sales price and harvest weight between the stocking periods.

To improve fish health management and reach the targets of reduced mortality and, consequently, increased revenue, the challenges and risk factors have to be identified. This underscores the need for close monitoring of important factors affecting fish health throughout production cycle. The key to sustainable production with healthy fish and low mortality is to be able to make the right decisions at the right time in order to allocate necessary resources to address the identified health challenges (Mardones, 2020).

5.1.3 Monitoring fish health

In animal health, monitoring has been defined as “the making of routine observations on health, productivity and environmental factors and the recordings and transmission of these observations.” (Thrusfield & Christley, 2018). Another term often used is “surveillance”. While closely related to monitoring, surveillance is a more intensive form of data recording with a predefined plan to mitigate disease

risk (Thrusfield & Christley, 2018). Monitoring is hence a broader and less specific concept than surveillance, which often targets specific diseases. In this thesis, which focuses on more than just diseases, the term monitoring is used throughout.

Monitoring can also have slightly different meanings depending on the context. Monitoring might refer to the recording of a certain factor (e.g., mortality, temperature etc.) or it could, as described in 4.1, refer to the more overarching monitoring of health in a population for which data from the detailed monitoring of individual factors are available.

5.1.3.1 What is monitored today?

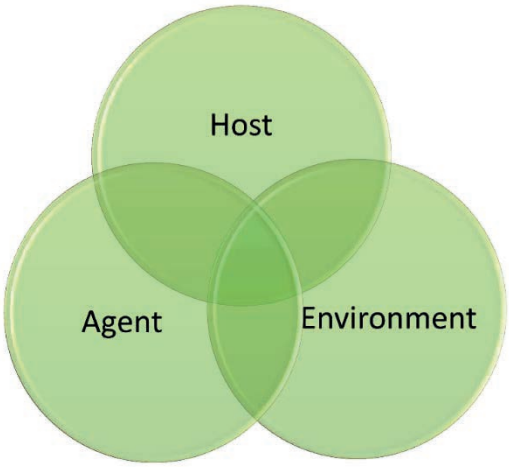

Figure 6. The figure is an adapted version of the epidemiological triad first described by Snieszko (1974). Illustrating the relationship between host, agent and environment.

Infectious disease, and eventually mortality, occurs when the interplay between host, pathogen and environment is out of balance. These interactions are often referred to as the "epidemiological triad" (Snieszko, 1974). In production animal husbandry, disease and mortality can occur also without a pathogen or infection. For example, technical failure, trauma or stressors introduced in the production management can cause damage, disease and mortality (Thrusfield & Christley, 2018). The original triad is therefore adapted, and "pathogen" is denoted "agent" in Figure 6, to account for the above-mentioned multitude of causes to disease. Regardless, factors affecting the epidemiological triad must be managed to keep the

fish healthy in this interplay. Every measure taken to improve the host's resilience to pathogens and disease (e.g., vaccination, genetics), prevent agents from entering the production environment (e.g., filtration and disinfection of water), or ensure an environment adapted to the fish are all preventive measures and part of fish health management. The challenge is to both identify what to monitor and untangle the causality (i.e., the relation of cause to effect), in this case how the monitored factor affects fish health (Blood, Studdert, & Gay, 2007; Mardones, 2020; Thrusfield & Christley, 2018).

Important fish parameters closely monitored at all Norwegian salmon farms, in all cages and tanks include daily mortality and feed consumption. Norwegian legislation also requires farmers to keep records of these parameters (Akvakulturdriftsforskriften, 2008). Several environmental parameters are also monitored (e.g., temperature, oxygen, salinity, turbidity, water current etc.), however, the frequency of measurement, as well as what is measured, varies between farms. Land-based facilities, especially those using RAS, also closely monitor water quality with several additional parameters (e.g., pH, CO_2, nitrogen compounds) daily (or more frequently) to swiftly detect changes in the environment.

Several infectious diseases are also monitored. Regular health visits by fish health personnel (monthly or bimonthly depending on size of farm) to all farms with fish in production is an important national preventive health measure in Norway (Akvakulturdriftsforskriften, 2008). The main aim of those visits is early detection of notifiable infectious diseases (e.g., ISA) by clinical assessment of fish throughout the production period (Forskrift om sykdom hos dyr, 2015). In addition, salmon lice and pancreas disease (PD) (Salmonid alphavirus) have their own surveillance programs in the legislation (Forskrift om lakselusbekjempelse, 2013; Forskrift om tiltak for å forebygge, begrense og bekjempe PD hos akvakulturdyr, 2017). For PD, the regulations require monthly sampling from dead or moribund fish in all marine farms. Salmon lice infestation levels are monitored in all cages and reported to the authorities weekly. Other infectious diseases are monitored voluntarily and vary by farm. Examples of such diseases and pathogens are amoebic gill disease (AGD, caused by *Paramoeba perurans*), piscine myocardiovirus (PMCV) and piscine orthoreovirus (PRV).

5.1.3.2 Monitoring mortality

For salmon farmers, mortality numbers are readily available and could be used in daily and strategic production management. Obviously, for the individual that has died, preventive measures are pointless at this stage. But when health is assessed in a population of production animals (e.g., poultry, swine or fish), mortality is a valuable tool to identify health challenges in production (Bang Jensen, Qviller, et al., 2020; Gåsnes et al., 2021; Heier, Høgåsen, & Jarp, 2002; Knauer & Hostetler, 2013). Nationally, mortality is the most important indicator of the health situation in salmon farming, even though mortality is considered a crude parameter when assessing health (Grefsrud et al., 2018; Noble et al., 2018; Sommerset et al., 2021).

However, mortality is an objective measurement which is easy to quantify and occurs only once (Schneider, 2002). Mortality in aquaculture is often communicated through a proportion, where the number of dead fish is the numerator and the number of fish in the population (e.g., cage or farm) is the denominator. This is also referred to as mortality risk (Toft, Agger, Houe, & Bruun, 2004). For example, if 100 000 salmon smolts were transferred to a cage ("population at risk") and 5 000 died before harvest, this would give a mortality of 5 % in this cage during the production cycle (5% mortality risk). To compare this mortality measurement between different populations, the time period for when dead fish are registered need to be comparable (e.g., production cycle) and the population closed (i.e., no fish enter or leave the population during the time period). Another commonly used mortality measurement is mortality rate, describing the number of dead fish over a defined time-period (Dohoo, Martin, & Stryhn, 2009; Toft et al., 2004). Mortality rate thus differ from mortality proportion by handling time within the calculation.

Median total mortality in the seawater phase in Norwegian salmon farming has been between 15-20% the past six years (Figure 7) and mortality in production represents a major challenge for the industry (Sommerset et al., 2021; Sommerset et al., 2022). However, mortality varies substantially between farms as indicated by the interquartile range (IQR) in Figure 7 (orange lines). The variation is even more extensive, since this imply that 25% of farms have mortality above and below those orange lines. Bang Jensen et al. (2020) found single farms with more than 75% mortality in one production cycle, emphasising the variation between farms. One limitation of data deriving from national records, where farmers report mortality monthly, is that data structure and reporting are not adapted to capture information based on the lowest epidemiological unit – the cage. Instead, data need to be

aggregated to farm level when used across companies and nationally (Bang Jensen, Mårtensson, & Kristoffersen, 2020; Bang Jensen, Qviller, et al., 2020). To better understand factors affecting mortality, and why mortality varies to this large extent, studies need to be performed on data at the lowest level possible and retrieved directly from farmers (Bang Jensen, Mårtensson, et al., 2020).

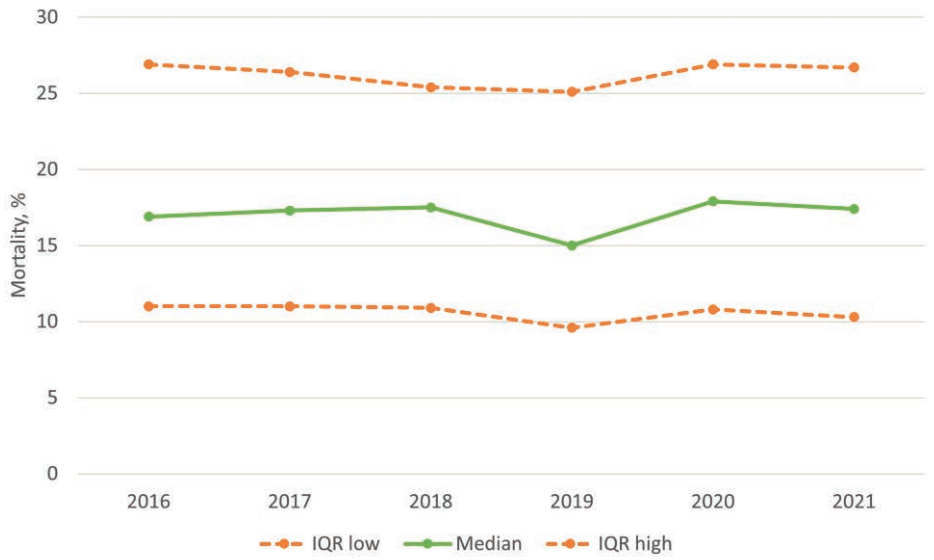

Figure 7. Graph of accumulated mortality of finished production cycles in Norwegian salmon farms by the year fish were harvested, based on the entire production cycle at sea. The green line shows the median mortality 2016-2021 and interquartile range (IQR) is included as "IQR low" for the 25th percentile and "ICR high" for the 75th percentile. Numbers originate from the fish health report published by the Norwegian Veterinary Institute each year (Sommerset, Bang Jensen, Bornø, Haukaas, & Brun, 2021; Sommerset et al., 2022)

Mortality records describe where and when fish die. However, if farmers aim to prevent mortality, the question should be why they die, or rather from what. Consequently, the cause of mortality must be identified to effectively use the information in production and apply appropriate interventions. In human medicine, cause-specific mortality classification is a well-established method of health monitoring. Cause-specific mortality classification is when you assign a cause to each death. Hence, mortality records are both quantitative and qualitative (Brooke

et al., 2017). The importance of collecting data on mortality dates back to the eighteenth century and works like *Nosologia Methodica* by Sauvages, *Genere Morborum* by Linnaeus and *Synopsis Nosologiae Methodicae* by Cullen (Knibbs, 1929). However, systematic international collaboration on a standardised list of mortality causes started at the First International Conference for the Revision of the International List of Causes of Death in August 1900, when delegates from 26 countries adopted the first list of causes of death (Knibbs, 1929; World Health Organization, 2021a). Today, 120 years of continuous international collaboration on mortality classification have resulted in the 11th revision of the International Classification of Diseases, to be implemented in 2022 (World Health Organization, 2019, 2021a). The standardisation makes it possible to extract data and aggregate it for a chosen level of detail, time and epidemiological unit. Exemplified in Figure 8, which shows mortality data reported to the WHO from Norway in 2016. This dashboard view gives information about the mortality causes registered in various comparisons.

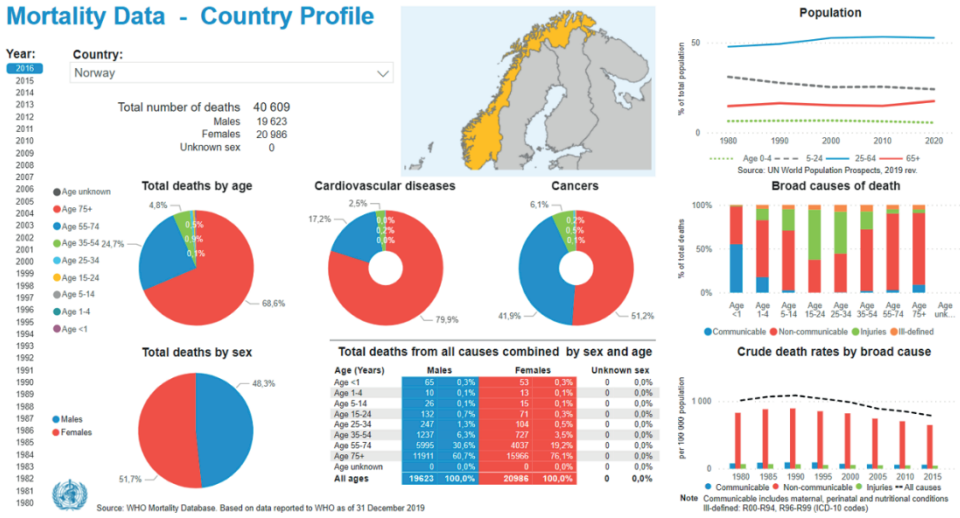

Figure 8. Example of visualisation of causes of mortality from human data (with permission, from https://www.who.int/data/mortality/country-profile)

The mortality data collected throughout the years in human medicine have continuously contributed to the development of health policy nationally and globally with the aim of improving the health of the human population and reducing mortality and morbidity (Naghavi et al., 2017; World Health Organization, 2021b).

Previous studies indicate there is potential for using cause-specific mortality registrations in salmon farming (Aunsmo et al., 2008; Nilsen, Nielsen, & Bergheim, 2020). This is beneficial primarily for the farmer as it provides information about causes of mortality otherwise only available through targeted studies of risk factors. A standard method of recording cause-specific mortality in the salmon industry would also increase the information available in national statistics, hence contributing to improvements at both local farm level and nationally when implemented in policy-making and research activities.

5.1.3.3 Monitoring factors to prevent poor fish health

The monitoring of factors related to health should preferably target early levels of prevention to prevent diseases or mortality in the population (as described in Figure 5). However, the level of prevention necessary would be considered differently depending on the viewpoint. If, for example, the notifiable infectious disease ISA is diagnosed at one farm in Norway, measures are taken to prevent further spread in the national salmon population (often by stamping out the fish at this farm). This kind of disease monitoring would be considered a form of secondary prevention from a national perspective. For the fish population at the diseased farm, however, preventive efforts would be considered a failure (or at best defined as tertiary prevention if a disease was treatable). This example illustrates the preventive paradox (Rose, 1981), which, when adapted to fish health, holds that "a preventive measure which brings much benefit to the population offers little to each participating individual farm" (Mardones, 2020).

Monitoring (and subsequent interventions) at the primary prevention level would also help the farm in the above example address the challenges of the preventive paradox. At the primary level of prevention, risk factors before disease development would be the target of the monitoring, giving the farmer an early warning. However, the challenge is to identify these risk factors and verify their causal association with the health outcome and to determine how to subsequently measure and monitor them.

The identification of risk factors, which enables intervention at the primary prevention level in aquaculture, is difficult. Apart from RAS facilities, environmental or pathogenic factors in the epidemiological triad (Figure 6) are almost impossible to control in fish farming (Tlusty, 2020). Land-based facilities have more opportunities to monitor the environment and detect pathogens. However,

monitoring environmental factors would be an indirect measurement if fish health is the target. This leaves risk factors attributed to the host, also called intrinsic factors (Thrusfield & Christley, 2018), as potential targets for monitoring of fish health, especially in the marine phase.

Salmon gills and mucous cells

One of the organs in closest contact with the water environment is the fish gill. In addition to respiration, the gills are responsible for several vital physiological functions: osmoregulation, excretion of nitrogenous waste, pH-regulation, hormone production and immune regulation (Evans, Piermarini, & Choe, 2005; Gomez, Sunyer, & Salinas, 2013). Figure 9 shows the delicate anatomical structures of the gill. Salmon has four gill-arches on each side (Figure 9A). Each arch consists of a bone structure rostrally and two hemibranchs, each with one row of filaments extending caudally from the bone (Figure 9B-C). The respiratory epithelium is lining lamellae protruding perpendicular to the entire length and on each side of the filaments (like a feather, Figure 9D) (Bruno, Noguera, & Poppe, 2013). The structure of the gills results in a large interface area to the water for exchange of gases, acid-base regulation, osmoregulation, and excretion of nitrogenous waste products.

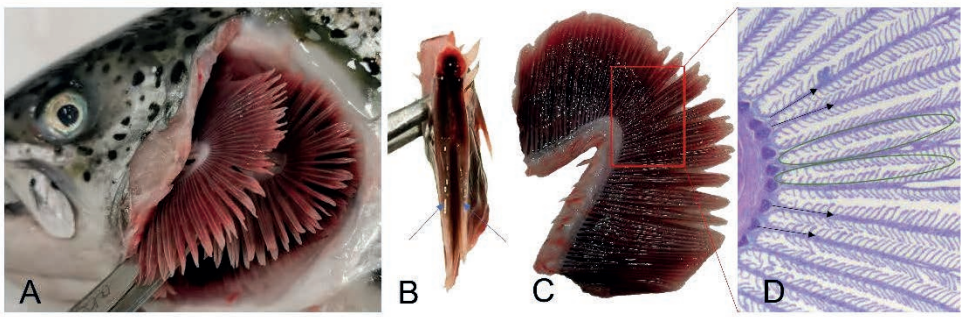

Figure 9**.** A: Gill arches *in situ*. B. Cross section of a gill arch, showing the two hemibranchs (blue arrows). C: Lateral view of one gill arch. D: Histology section of gill tissue (red box in C indicates the area), selected filaments are marked with black arrows and lamellae are protruding perpendicularly from the filaments (indicated with green circles). Photo credit: Alexander Figenschou (A-C), Håvard Bjørgen (D).

The gill epithelium is also an important part of the immune system, acting as a first line of defence against potential pathogens or harmful agents in the water

environment (Bjørgen & Koppang, 2021; Koppang, Kvellestad, & Fischer, 2015; Rodger, 2007). A mucus layer consisting of glycoproteins (mucin) and water lines the apical surface of the epithelium. Sometimes the mucus layer is referred to as an extracellular biofilm, since it also harbours the host microbiome which interact with the immune system (Ferguson, 2006; Llewellyn, Boutin, Hoseinifar, & Derome, 2014). Mucus covers all epithelial surfaces in the fish, that is gill, skin and gastrointestinal tract (Gomez et al., 2013; Shephard, 1994). Gill mucus is primarily produced by the mucous cells, found in the base of the lamellae but also scattered in the entire length of the lamellae (Ferguson, 2006). Production of mucus increases when gills are irritated by exogenous agents (Ferguson, 2006; Koppang et al., 2015). Ferguson (2006) identified three initial interactions between the gill and a disease-causing agent. Two of the interactions are described as external, by either colonization of the extracellular mucus layer, or waterborne toxins affecting the epithelial cells. This highlights the role of the gill epithelium as a primary barrier of the immune system. Ferguson (2006) also outline the connection between mucus production and stress. This was further elaborated by Llewellyn et al. (2014), discussing the importance of stress since it alters the composition of mucus and reshape the gill microbiome.

Early stages of several immune responses are thus occurring in the mucosal layer of the gills. If the aim is to intervene at the primary prevention level (Figure 5), it would be favourable to monitor those responses. One parameter previously used to assess acute gill responses in experimental settings is the number of mucous cells (Ferguson, Morrison, Ostland, Lumsden, & Byrne, 1992; Haddeland et al., 2021; Roberts & Powell, 2003; Speare, Arsenault, MacNair, & Powell, 1997). Monitoring of the number of mucous cells would target early stages of the immune response in one of the first organs to encounter potentially hazardous agents, resulting in monitoring of a host response before the fish is diseased. If successful, this would give the fish farmer an early warning about the health status in the population. However, there is scarce information about the variation in mucous cell numbers between healthy, individual fish, and between fish populations in commercial farms.

Gill mucus microbial composition

The gill mucosal surface further regulates a complex interplay with the microbiota that live in symbiosis with the host at the border between the host and the surrounding environment (Gomez et al., 2013; Legrand, Wynne, Weyrich, & Oxley, 2020). Microbiota refers to the microorganisms present in a defined environment

(water or the mucosal surface of the gill, for example). The composition is commonly characterised through analyses of biological samples with molecular methods, such as sequencing of marker genes. In aquaculture, the method most frequently used is 16S rRNA gene sequencing for detection of bacteria and other prokaryote organisms. The DNA-sequences generated are subsequently assigned a microbial taxon at different taxonomic levels (Marchesi & Ravel, 2015). This generates extensive information about the species richness and abundance of bacteria present in the sample investigated. In aquaculture, microbiomes in the production environment and the host have been studied and described extensively during the recent years (Fossmark, Attramadal, Nordøy, Østerhus, & Vadstein, 2021; Legrand et al., 2020; Minich et al., 2020). The mucosal tissue of the intestine, skin and gills are the most frequently sampled areas of the host, generating knowledge characterising the taxonomic diversity of each region. Our understanding of the interactions between microbiome and host and how to manage microbial ecology in aquaculture is still limited though (Legrand et al., 2020; Llewellyn et al., 2014). However, if the relation and interactions between microbiome, fish and production environment are described and disclosed, the potential for monitoring fish health and fish production by this tool would be substantial.

5.1.3.4 Knowledge gaps

In 2021 the mortality in Norwegian salmon farming reached 54 million individuals and the annual mortality has been 15-20% for the last six years, as seen in Figure 7. The rapid growth and industrialisation of salmonid aquaculture production have not been adequately developed to secure the good health and welfare of farmed salmon. This situation is not sustainable, for the fish, the farmers or society. Improvements to the production process should thus aim to produce fish with increased resistance to disease and able to cope with handling. At the same time, it is also necessary to identify the best preventive measures to reduce negative impacts of existing treatment methods and disease. Identifying effective preventive measures requires detailed knowledge about factors affecting health and mortality.

The lack of robust monitoring methods limits the potential to identify fish-groups at risk of deteriorating health, disease or increased mortality at crucial life or production stages in salmon farming.

5.2 Aim of the thesis

The aim of this PhD thesis was to investigate monitoring methods that can be used to improve fish health management in salmon farming.

The following specific objectives were defined:

1. Analyse mortality patterns in salmon farming using cause-specific mortality classification (Paper I)
2. Suggest a standard for classification of cause-specific mortality in salmon aquaculture (Paper II)
3. Explore gill mucous cell numbers and gill mucus microbial composition as indicators to monitor gill health status (Paper III and IV)

5.3 Materials and Methods

5.3.1 Conceptual framework

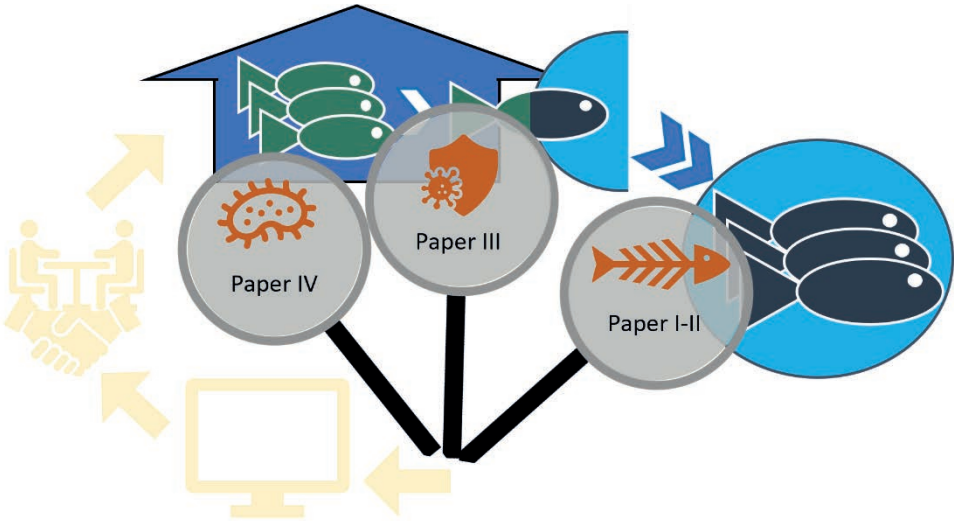

Figure 10. The conceptual framework of this thesis in the context of fish health management. This thesis has explored monitoring of three factors related to health in different parts of the production cycle: gill mucous cell count (Paper III) and gill mucus microbial composition (Paper IV) in the last part of the freshwater production, before sea transfer, and cause specific mortality classification (Paper I-II) in the marine phase.

The conceptual framework to investigate fish health monitoring methods was built by two approaches. Cause-specific mortality classification represented the first approach (Figure 10, I-II), and the gill was the target of the second approach (Figure 10, III-IV). Hence, the first approach was to investigate mortality patterns in salmon production using cause-specific mortality classification (Paper I) and subsequently suggest a standard and means of implementing this tool in production (Paper II). The second approach was accomplished by sampling salmon from different commercial hatcheries and study factors associated with the first line of defence in the immune system: gill mucous cell count (Paper III) and gill mucus microbial composition (Paper IV). Overall, the studies seek to answer the aim to investigate monitoring methods that can be used to improve fish health management in salmon farming.

5.3.2 Monitoring mortality (Papers I-II)

5.3.2.1 Mortality patterns (Paper I)

Material

Data from the production management systems from two farming companies were used in the study. Daily production data from all fish transferred to sea the years 2017 and 2018 were retrieved. Variables of interest were mortality and cause-specific mortality registrations for each cage, as well as risk factors for mortality related to transfer of fish between the hatchery and the farm at sea, in addition to events including handling of the fish during the marine phase. Data were retrieved at cage level. Information about the fish-group delivered to the marine farms from the hatcheries upon transfer of the fish were retrieved through "smolt documentation sheets" from each fish-group. These sheets had to be gathered as PDF files stored locally at farming sites, as opposed to the data collected from the production management system which could be retrieved from a central database at each company. The intention was to gather detailed information from the hatchery for each fish-group from the smolt documentation sheets. Unfortunately, the information recorded in the documents was fragmented and not standardised and varied across the different fish-groups.

Methods

Comprehensive descriptive statistics were important for visualising the distribution of mortality causes throughout the production cycle. In addition, cumulative mortality at 180 days post transfer and at harvest were used as outcomes in a cross-classified multilevel regression model. The cross-classified structure was needed to incorporate the hierarchical structure of salmon production where farms at sea receives smolt from multiple hatcheries, with fish-group nested within farm. The focus of the analysis was the sources of variation within the farming hierarchy. There were limited numbers of units included in the study, and therefore the measured effect of each predictor was difficult to extrapolate from the study population. However, the external validity of the sources of variation results was assessed to be robust.

5.3.2.2 Cause-specific mortality classification (Paper II)

Material and methods

In Paper II the development of a proposed standard for classifying mortality into cause-specific categories was described. The first part of the study was an assessment and description of the current situation in terms of mortality classification systems within aquaculture, land-based animal production and human medicine, as well as the relevant literature. Identical to the human mortality classification system (World Health Organization, 1979), we settled on using the underlying cause of death also when classify mortality in salmon farming. The underlying cause is the factor that starts the "train of events leading to death", and thus the causal factor initiating the pathophysiological events that eventually cause death (Brooke et al., 2017). Further, the salmon farmer was identified as the primary user of information generated from the system. This underscores the importance of causality in the system since the farmer can act upon information regarding identified events causing mortality and work preventively based on this knowledge. The second part of the study aimed to establish a list of mortality causes for use in salmon farming, and how to implement the classification system into the farm management. The initial draft of the classification system and its intended use was inspired by the system used in human medicine to classify causes of mortality, namely the International Classification of Diseases, for mortality and morbidity statistics (ICD) (World Health Organization, 2019). This work also included a survey among some salmon farming companies about which causes of death they recorded, personal experiences from the authors and input from a meeting where a draft of a list was presented to about 70 fish health personnel.

5.3.3 Monitoring gill health status (Paper III and IV)

Material

Salmon (n=220) were sampled from six commercial salmon hatcheries, five RAS (RAS I-V) and one FT farm (FT I), at the production stage between vaccination and transfer to sea. Gill histology samples from all fish were included in a study of variations in gill mucous cell numbers (Paper III). In addition, swab samples from fish gill surfaces (n=160) and environmental samples (water [n=24] and biofilm [n=24]) from a subset of the farms (RAS II-V) were used to investigate microbial composition (Paper IV). An overview of the fish samples deriving from each farm in relation to each study is shown in (Figure 11).

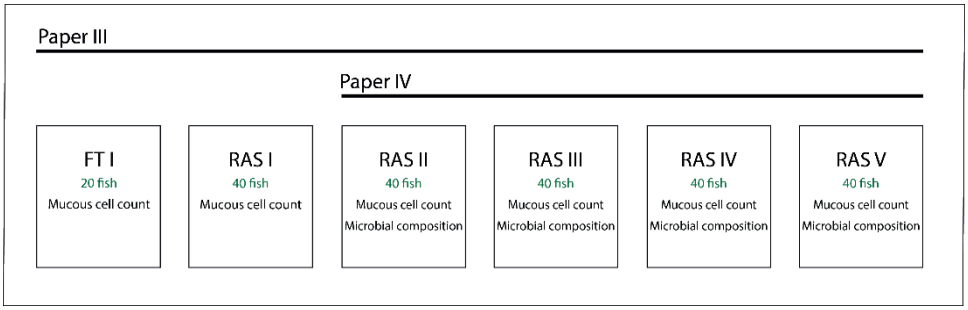

Figure 11. Overview of included farms, studied outcomes and number of fish sampled in Papers III and IV.

5.3.3.1 Gill mucous cell count (Paper III)

Method

Gill samples were processed for histology through standard procedures, including staining with haematoxylin and eosin and periodic acid-Schiff (PAS) for detection of mucins (Bancroft & Gamble, 2008). A counting method of mucous cells was developed and implemented as a part of the study. The method built on previously published studies in which mucous cell count had been used as an indicator of gill damage (Ferguson et al., 1992; Speare et al., 1997). However, the method was not described in detail in those studies, making it difficult to reproduce. We developed criteria to make the counting of mucous cells more objective, and this improvement was described and evaluated. Mucous cells from the sampled fish gills were counted according to the developed method and included as an outcome in a regression model, with “fish length” and “farm” as predictors.

5.3.3.2 Gill mucus microbial composition (Paper IV)

Method

Extracted DNA from gills and biofilm swabs and water samples were amplified targeting the variable regions 3 and 4 of the preserved 16S rRNA bacterial gene and sequenced (Illumina MiSeq platform). Subsequent microbiome data analyses were performed in QIIME2 (Bolyen et al., 2019). In addition, analysis of bacterial and host DNA abundance in the extracted gill DNA was performed by absolute quantification of 16S rRNA (bacteria) and 18S rRNA (host) gene copy numbers respectively, using Naica Crystal digital PCR (dPCR) system (Stilla Technologies). Alpha microbial diversity was calculated with Shannon index as the metric of choice. A combined

grouping variable (farm-tank-sampling timepoint) was used as random effect exploring sources of variation in a two-levelled regression model with Shannon index of gill microbiome diversity as an outcome.

5.4 Results

5.4.1 Mortality patterns (Paper I)

Results indicate that causes of mortality to a large extent vary with time in production. Smolt-related mortality was the most frequent cause of death registered up to 180 days post sea transfer. Overall accumulated mortality at harvest identified causes related to handling to be the dominant cause of death. The third group of causes in the study, infectious diseases, was the second most frequent cause of death at both time-points. The variation was also evident between the fish-groups, with both the causes of death and numbers of dead fish varying widely. The fish-group was the most important source of variation when included as a level in the cross-classified multilevel model, indicating the importance of focusing on each fish-group when strategically working to reduce mortality in the industry. Information deriving from cause-specific mortality classification could further help farmers to identify drivers of mortality in their own production.

5.4.2 Cause-specific mortality classification (Paper II)

The proposed mortality classification system was constructed in a hierarchical structure with three levels (Level 1-3) identifying the underlying cause of death. At the lowest level (Level 3) of the hierarchy, every underlying cause was given a unique alphanumeric code. Causes were grouped by causality in subcategories at Level 2 and in six main categories at Level 1 with each cause belonging to only one subgroup and one main category. The Level 1 categories were infectious diseases, losses caused by environmental impacts, injuries or trauma, physiological causes, other causes, and unknown causes. The hierarchical structure makes the system flexible as it allows for registration of causes of mortality at any level and for data extraction to be aggregated at the desired level. Therefore, out from ambitions each farmer can decide the appropriate level of detail for his production, while the standardised format makes it possible to compare results across companies, regions or nationally.

5.4.3 Gill mucous cell count (Paper III)

Gill mucous cell count varied across the six farms and individual fish. There was clearly an individual effect mediated by the size of the fish. However, when this was included in the regression model as a predictor together with “farm”, the proportion of variance in mucous cell count explained by the model (R-squared) was twice as high compared to when only size (fish length) was included indicating a variability

in mucous cell count depending on farm-related factors, including when size was accounted for.

5.4.4 Gill mucus microbial composition (Paper IV)

Different microbial compositions were found between gills and the environment (water and biofilm). There were generally low levels of bacterial DNA detected in the gills and substantial differences between the farms. Bacterial DNA was also found to be negatively correlated to the amount of total DNA in the samples. The model attributed only 10% of the variation to the combined grouping variable, indicating that most variation in microbial Shannon diversity was attributed to the individual fish.

5.5 Discussion of findings and methods

Different methods of monitoring fish health have been studied in this thesis. Monitoring fish health by identifying the cause of mortality for each dead fish will help farmers identify losses in production and subsequently benefit fish health when the knowledge is integrated in the fish health management at the farm (Paper I and II). If implemented, this would contribute to the primary level of prevention. The methods of gill health monitoring by mucous cell count and microbial diversity (paper III and IV) are premature in comparison and further studies are needed to be able to provide farmers with useful information regarding fish health status.

5.5.1 Mortality patterns in salmon farming

Paper I shows the potential for using cause-specific mortality data both in a timeline of production and for each fish-group. Having an overview of mortality causes throughout the production period contributes to understanding when in production mortality occurs and what the dominating causes of mortality are across the included fish-groups. The variation in mortality between fish-groups was shown to be extensive in Paper I, showcasing the importance of information at the lowest population unit possible to identify causes of mortality. In itself, the major variation in mortality between fish-groups is essential knowledge, especially since the mean mortality at the farm level could be strongly influenced by single fish-groups with high mortality. Causes of mortality specific to groups with high mortality would be of primary concern for efforts to reduce mortality, and thus important to identify.

Another finding is the structural challenges the salmon farming industry are facing in terms of data flow in the production (Paper I and II). The results of Paper I clearly attribute most variation in mortality to the fish-group when used as a source of variation in regression modelling. The results in Paper II further underscore the importance of fish-group as the lowest population unit when working with mortality classification. However, data registration is based on the cage rather than fish-group within the production management system, making it difficult to follow the fish throughout production, for example if they are moved or split into more cages (Figure 11, fish-group C). Data would become more accessible, including for individual farmers, if this identification of fish-groups would become more uniform throughout and between production cycles.

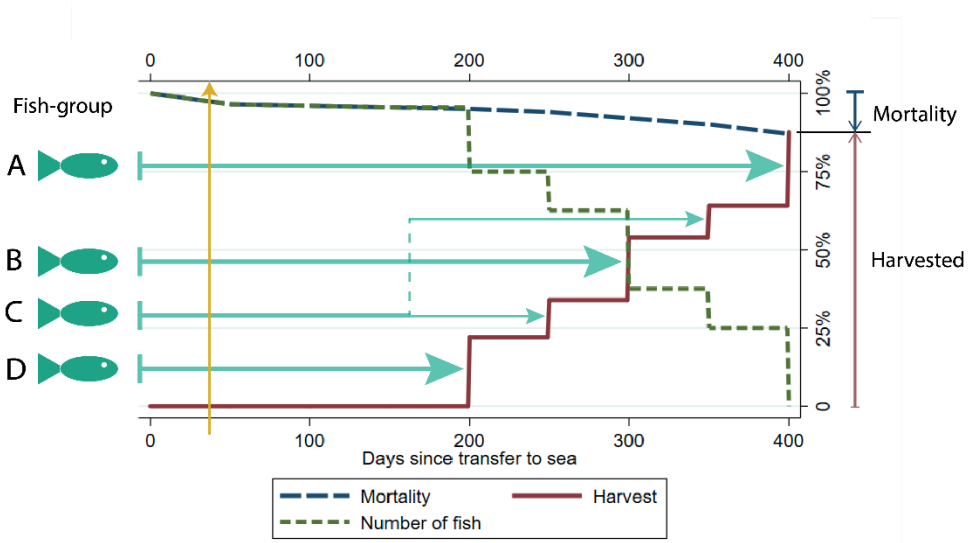

Figure 12. Illustration of a production cycle in a farm with 4 fish-groups (A-D). The fish was transferred to sea to four cages at day 0 and followed until harvest. Fish-groups A-D were slaughtered at different times during the production, indicated by the green arrows (and increase in proportion harvested, red solid line). Fish-group C was split in two cages at around day 175. Accumulated mortality during the production is indicated with the blue dashed line. The green dashed line represents the number of fish during production. Assuming no losses other than mortality or harvest, this decrease is equal to mortality and harvest during the production cycle. The red solid line is the proportion of fish harvested during the production cycle. The yellow vertical line illustrates how daily mortality is calculated, including all fish-groups in the dataset in the measurement

5.5.1.1 Mortality measurements

In an attempt to utilise all mortality data gathered for Paper I, data from all fish-groups (including mixed or split groups) were included in a descriptive part. Daily mortality was the sum of mortality each day (across all cages) in the entire dataset, divided by the total number of fish in all fish-groups each day. Referring to Figure 12, this calculation is illustrated by the vertical yellow arrow, measuring mortality as a proportion across the number of fish in all fish-groups each day. Total mortality was in Paper I also defined as proportion: the sum of dead fish in the entire

production cycle (in all cages), divided by the total number of fish transferred to sea. In Figure 12 this proportion is 12.5%, indicated with the blue arrow (which equals mortality risk in a production cycle at farm level). These two measurements (daily and total mortality) work for descriptive purposes and to visualise mortality patterns, however, it becomes insufficient when analysing risk-factors varying in time and between fish-groups. Here the cumulative mortality for each fish-group was used, and in Figure 12 this would represent summarising mortality horizontally, along the green arrows for each fish-group, excluding split groups at the timepoint of splitting. This equals the mortality risk in each fish-group (in a production cycle).

5.5.1.2 Traceability of data

The production management systems in salmon farming today have no standardised way of defining a fish-group or following one as a unit, which is important when investigating biological challenges. Hence, fish-groups must often be traced manually when studying risk factors retrospectively. The lack of a standard system for tracing fish-groups within the farming industry is a constraint for effective management of production and an unnecessary obstacle, which should be addressed rapidly. Paper I further investigated risk factors associated with events occurring at different times during production cycle (e.g., lice treatments). The splitting and mixing of fish-groups makes it challenging to follow fish-groups as an epidemiological unit as risk factors may affect parts of the unit differently (Figure 12, fish group C). Hence, those fish groups had to be excluded from the study in Paper I. In this study, more than 40% of the fish-groups were excluded at the time of harvest due to traceability challenges.

Assessment of fish health at the fish-group level also needs to be structured and more widely accessible to production management than it is currently. Reports from veterinary health visits are often stored as written documents, which are difficult to transform into information that is applicable to management systems (Paper II). This argument is also relevant to all aspects of production involving procedures potentially affecting fish health (e.g., well-boat operations, treatments, smolt documentation sheets etc.). Several aspects of health assessment are therefore lost in production management today (Paper I). This could be improved if information was incorporated in the management systems more extensively.

5.5.2 Standardisation of mortality classification

Regarding the applicability of the mortality classification described in Paper I, cause-specific mortality is already used by several farming companies today. However, each company has their own approach to classifying and labelling causes of mortality (Paper I-II). There is some overlap between these, of course, but since the same cause can be labelled differently, comparisons between companies are challenging (Paper I). A positive aspect of a systematic approach to registering mortality classification based on causality is the farmers' ability to identify causes of mortality themselves. Since the system is designed to target the underlying cause, the data can be used instantly to inform farmers about the production continuously and take relevant measures to prevent the major causes of mortality identified. Quantification of mortality alone would not identify any causes, and to investigate possible causes of mortality, studies of several different data sources would be needed to find relevant risk factors (Paper II). Hence, the concept of the underlying cause when registering cause of death is a major advantage, for farmers especially, in managing fish production.

The ICD used in human medicine has unique codes for each cause of death, and the system is structured in a hierarchical manner to group similar causes of death together (World Health Organization, 2019, 2021b). Salmon farming does not have this standardised system or defined groups of causes. The standard proposed in Paper II would thus improve the communication and dissemination of results from classification work and make it more accessible, thus improving production, at least when one considers that the raw data already exists as pointed out in Papers I and II. In Figure 13, the potential in grouping cause specific mortality data from salmon farming is visualized, identifying the proportion of each grouped mortality cause within the cumulative mortality at harvest, for each fish-group (Paper I). If such structure of cause specific mortality is implemented, information about causes of mortality could be accessible at the farm to inform about causes of death in production – in a similar manner as displayed by the dashboard view in Figure 8.

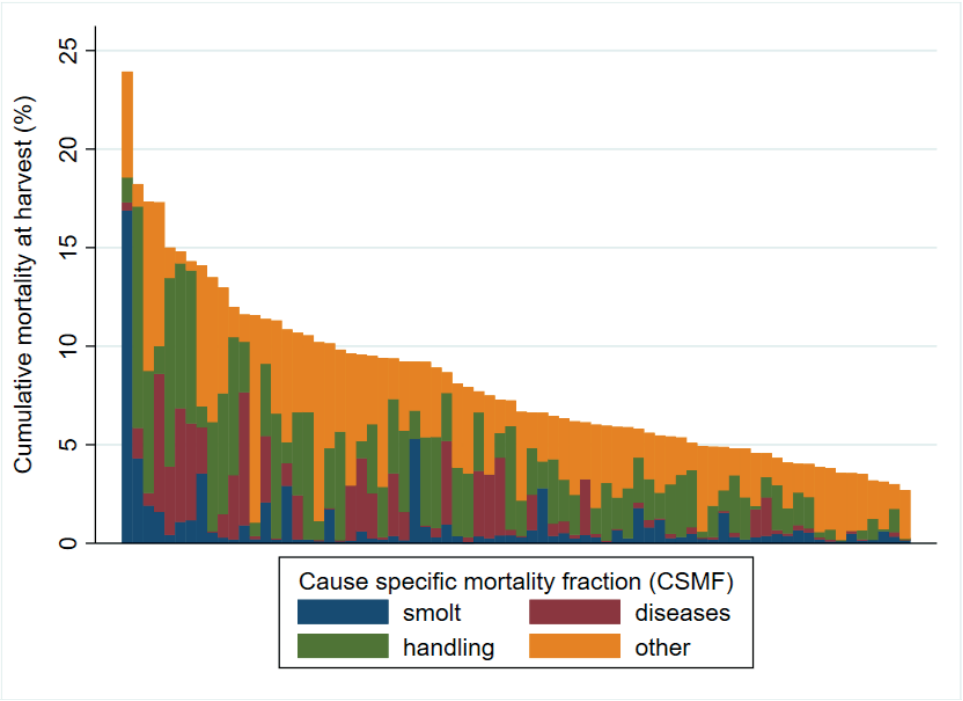

Figure 13 From Paper I, illustrating causes of mortality in salmon farming grouped under "smolt", "diseases", "handling" and "other", displayed as part of the cumulative mortality at harvest for each fish-group (n=74)

A unified definition of a fish-group might also improve the national statistics on salmon health in Norway. Existing legislation requires farmers to report mortality data at cage level monthly (Akvakulturdriftsforskriften, 2008). However, since fish may change cage throughout the production cycle, the data need to be aggregated at farm level when official mortality records are used in reports or epidemiological studies (Bang Jensen, Mårtensson, et al., 2020; Bang Jensen, Qviller, et al., 2020; Oliveira, Dean, Qviller, Kirkeby, & Bang Jensen, 2021). This effectively masks variation between fish groups, which was extensive according to Paper I.

The different epidemiological studies in this thesis were built on either primary observational data (Papers III-IV) or secondary data from the production management system (Paper I). A common challenge in all these studies was to establish causality, namely attempting to identify causal relationships between production parameters and the outcomes of either mortality (Paper I), mucous cells (Paper III) or microbial composition (Paper IV). It is challenging to isolate the effects

of specific production parameters on the outcome. For example, for the outcome cumulative mortality at 180 days in the regression model described in Paper I, the predictors "temperature in sea at transfer", "days in hatchery" and "stocking period" were shown to interrelate. Investigating the causal relationship shows that all these variables describe a similar time-point in production. Sea temperature is correlated with time of year, the number of days in hatchery will to some extent correlate to the time of year when fish are transferred to sea, and time of stocking is also strongly associated with time in production. Hence, the challenge is to find the causal pathway to mortality, differentiating between what is actually measured with the predictor from possible confounding effects (e.g., time in this case).

5.5.3 Monitoring of gill health status

In Paper III, gill samples from more than 200 clinically healthy salmon were examined by histopathology, and no major pathological changes were found. This indicates fish reared in those facilities had good gill health. However, if the aim is to monitor gill health to prevent diseases, detection of variation in apparently healthy gill tissue would be preferable. In Paper III, the results show variation in mucous cell counts between the investigated fish, as well as between farms. The latter difference is important since it points to farm-related factors (e.g., management, water quality etc.) affecting the number of mucous cells. However, it is necessary to establish a baseline for this outcome to further identify values representing deteriorating health. This would be equivalent to the concept of population-based reference interval, widely used in human and veterinary medicine for a range of diagnostic parameters commonly used in clinical decision-making processes (Friedrichs et al., 2012). Such reference intervals are needed if mucous cell count should become an applicable monitoring tool.

To detect an early immune response measuring the amount of produced mucus, or mucins, could be an alternative approach to counting mucous cell. Several methods to measure airway mucus and mucins has been developed in human medicine (reviewed by Atanasova and Reznikov (2019)). For example molecular methods such as RT-PCR (Guzman, Gray, Yoon, & Nettesheim, 1996) and northern blot (Chen, Nickola, DiFronzo, Colberg-Poley, & Rose, 2006; Zuhdi Alimam et al., 2000) in addition to protein detection by ELISA (Lin, Carlson, St. George, Plopper, & Wu, 1989). These methods detect specific mucins within the mucus, and knowledge about the mucin composition and their properties in the given species is therefore needed. Atanasova and Reznikov (2019) further points towards the sampling

procedure as an important factor to consider when investigating mucus characteristics in the human airways. As with most other sampling procedures, a lack of standardization may influence the results. They conclude that histological imaging remains the gold standard in detection of goblet cell hyperplasia and that RT-PCR can be used to detect changes in expression levels of mucin and regulation of the production.

Recent publications have investigated fish mucus and mucous cells using various methods. Marcos-Lopez et al. (2018) studied gene expression in salmon with AGD to investigate regulation of mucin production. Benktander et al. (2020) found that gill mucins are more complex compared to mucins in skin using liquid chromatography–mass spectrometry. Publications from Pittman et al. describes a stereology-based method that includes the distribution and size of the mucous cells in addition to the count (Dang et al., 2019; Haddeland et al., 2021; Pittman et al., 2013; Pittman et al., 2011). This has resulted in the development of a commercially available method to assess quality of the mucus barrier (www.quantidoc.no). The book "Systemic pathology of fish: a text and atlas of normal tissues in teleosts and their responses in disease" also discussed the use of mucous cell quantification and suggests a morphometric index based on numbers of cells per lamella to compare groups of fish (Ferguson, 2006). Tartor et al. (2020) studied different methods to sample mucus for analyses of specific immune-proteins. They found that a minimally invasive method of absorbing skin mucus showed satisfying results for immunoglobins compared to other sampling methods (scraping and wiping). Absorption was also tested on gill mucus, however, when compared to serum immunoglobulin content the correlation was weak.

The referred publications studying the mucus itself are seemingly limited to describe the content within the mucus (e.g., mucin, immunoglobulins etc), they do not quantify the actual production. The potential exception is the RT-PCR developed on human mucins, however, this has to be adapted to fish and relevant target proteins must be identified if to be adopted for aquaculture. In terms of developing a high throughput monitoring method, a PCR-method is obviously preferable. Especially if this can be performed with non-lethal sampling techniques (Tartor et al., 2020). The challenge is to identify what the PCR-analysis should detect in the mucus and correlate the targeted protein with the gill mucus production and overall health status.

In the present time, if the aim is to estimate the capacity of mucus production, it seems the available methods are limited to quantification of the number of mucous cells by histopathology. In Paper III, the method was based on the counting methods described in previous published experimental studies (Ferguson et al., 1992; Speare et al., 1997). One objective in Paper III was thus to further develop this method to define anatomical boundaries for where to count the mucous cells in order to increase reproducibility.

In paper III, the outcome of interest (number of mucous cells) was compared to the sight depth, measured by Secchi disc (Tyler, 1968). A wide range of water quality parameters are measured in a hatchery (e.g., turbidity, suspended solids, dissolved particles etc). However, it is challenging to compare results for water quality parameters as measurement methods are not necessarily comparable across farms. Due to limitation in available resources in the project (Paper III), but still with an ambition to describe water quality, it was attempted to assess particle content in the water using Secchi disc. The method is based on lowering a white disc and measuring the depth when it no longer is visible. Sight depth is thus a very rough method of estimating particles in the water, since it simply is based on the water transparency. It is not necessarily a relationship between reduced sight depth and poor water quality. However, this method is widely used in the sea to estimate the amount of algae and other organic particles in the water (Arup, 2002), which in turn potentially have impact on the fish (e.g., harmful algae bloom) (Rodger, Henry, & Mitchell, 2011). Sight depth measurements is cheap and very easy to perform in the marine phase. However, the Secchi disc was not found to be an appropriate method in the hatcheries, and future studies should invest in a better method to estimate water particle content.

Fish from four of the RAS-hatcheries included in Paper III were also sampled for gill mucous with swabs to perform 16S rRNA gene sequencing analyses (Paper IV). The result in terms of successful sequenced samples was poor, with only 40% of the samples successfully sequenced. However, the success rate strongly varied by hatchery: one hatchery (RAS II) had sequence results from all 40 fish and another only from four. Identical sampling procedures were performed in all farms. Investigations of the total amount of DNA and the number of 16S rRNA gene copies in the samples revealed that RAS II had the lowest level of total DNA but the highest number of 16S rRNA gene copies. Samples from the other hatcheries had higher total DNA and, correspondingly, a very low number of 16S rRNA gene copies.

Indicating low abundance of bacteria in gill mucous in general across the hatcheries, even though RAS II stood out with relatively high abundance compared to the others. Interestingly, this coincided with RAS II being the only hatchery with continuous production of fish. Continuous production meaning fish were stocked continuously, resulting in a stable biomass of fish in the RAS-unit. The other hatcheries practiced all-in-all-out, meaning the fish tanks were emptied and fallowed simultaneously between stockings for the entire RAS-unit. These findings indicate a higher abundance of bacteria in RAS systems with continuous production. When comparing the microbial composition between hatcheries, beta diversity (quantifying dis-similarities between samples) showed a distinct clustering of samples from RAS II and dissimilar from samples from the other hatcheries when those were considered as one group. However, while the results indicate this difference between RAS II and the other hatcheries, the effect on fish health remains unclear.

Even though the final dataset in Paper IV consisted of more than 60 samples, only four farms were included in the study. This makes conclusions based on a single explanatory farm-level variable challenging. The time frame for sampling could have been optimized, as sampling timepoints varied between post vaccination to just before sea transfer. The fish goes through a considerable physiological development (including smoltification) in the period between vaccination and sea transfer. Some of this could have been accounted for by registration of morphological traits separating for instance parr from smolt at sampling (Folmar & Dickhoff, 1980). This was not performed, and the only variable recorded for each individual (other than the outcome) was fish size (weight and length). Fish length was utilized in Paper III to account for fish size when modelling the number of mucous cells. The microbial composition investigated in Paper IV did not show a relationship with weight, and since the other explanatory variables were recorded at farm or tank level, the study therefore sought to investigate and describe where the variation resides in terms of level in the production hierarchy. Unfortunately, a more detailed analysis of relations between outcomes and specific explanatory variables was not suitable due to the low number of farms included, even though some production parameters varied (e.g., biomass, salinity and number of fish). In future field-studies, an appropriate number of farms and sampling points should be included to investigate the effect of such specific production parameters on the microbial composition and number of mucous cells.

One finding attributed to the individual fish in Paper IV was the variation in microbial diversity, assessed by the Shannon index (an alpha diversity metric), when investigated as a source of variation. This indicates that the host microbiome of these healthy fish gills modulates independently of the surrounding environment. Similar findings have been described for wild perch (*Perca fluviatilis*) (Berggren et al., 2022) and in a study investigating the skin-mucous microbiome of fish with ulcers in a marine farm (Karlsen et al., 2017). However, other studies claim the environment is the main driver for alterations in microbiome composition (Uren Webster et al., 2020). The inconsistent conclusions from different studies emphasise the challenges of establishing microbial composition as a tool for monitoring fish health.

The studies of mucous cell count and microbial composition in gills (Papers III-IV) point to a common challenge identified across several of the studies described above, namely, how to establish a causal relationship between the indicator measured and health status of the fish.

5.5.4 Methodological considerations

In cause-specific mortality classification, causality is built into the system at the time of registration since the underlying cause of death is the foundation for the classification system (Paper II). In addition, the data include records from all dead fish in the production. There is no extrapolation of results from a selection of individuals (study sample) from the study population, just compiled information from the entire study population. Obviously, a major potential pitfall of the entire system of cause-specific mortality is the uncertainty introduced by the person deciding the cause of death. This is a subjective assessment and thus, the precision of mortality classification systems can be questioned and needs to be addressed. In developing countries, causes of human mortality are often determined by information retrieved by interviews with relatives of the deceased rather than from hospital records or autopsy, as is common in the wealthy part of the world. The interview technique is commonly referred to as “verbal autopsy” (VA) (Soleman, Chandramohan, & Shibuya, 2006; World Health Organization, 2011, 2016). In salmon production, the method of establishing cause of death resembles this technique when the cause of death is determined based on information related to production (e.g., weather, treatments, events etc.) rather than autopsies of individual fish (Paper II). In human mortality classification, studies show a variation in precision among different methods used to classify causes of mortality

(Hernández et al., 2011; Murray, Lozano, Flaxman, Vahdatpour, & Lopez, 2011; Quigley, Chandramohan, & Rodrigues, 1999). However, there is also variation in precision based on the nature of different causes of mortality. For example, causes like road traffic, homicide and drowning were easier to correctly classify than poisonings, cardiovascular disease and pneumonia in a study on human mortality classification (Lozano et al., 2011). Thus, according to this study, mortality as a consequence of an event is more likely to be classified correctly with the VA method. Since VA is based on information received from interviews with relatives, rather than investigation of the actual deceased, an event would be easier to identify than a more diffuse cause like poisoning for example. These findings are advantageous for salmon production. Because mortality in salmon production is to a large extent event-based (Ellis, Berrill, Lines, Turnbull, & Knowles, 2012), there is reason to believe that a classification system based on similar principles as VA used in human medicine would perform well, especially when it comes to identifying mortality caused by events in production (Papers I and II).

A monitoring method needs to yield reliable, robust and reproducible results. The method of counting mucous cells in Paper III was developed with this in mind. It includes strict exclusion and inclusion criteria for each gill histology sample and has defined anatomical boundaries of where to count mucous cells. However, the method needs further validation. Findings from the study identify fish size as an important factor, and if the anatomical boundaries used when counting the mucous cells vary with size, this would introduce bias in the results.

Microbiome analyses by 16S rRNA gene sequencing methods, used in Paper IV, are challenging when it comes to validity and reproducibility. Sinha et al. (2017) investigated this by using identical samples which they sent to several laboratories, as well as using different protocols for analysis. The conclusion of the study was that each step in the analysis introduced variation of comparable effect size to that of the measured biological differences. It was further found that meta-analyses across microbiome studies are challenging because of this variation, and individual experiments frequently include incompatible protocol variables (Sinha et al., 2017). A meta-analysis of swine gut microbiota comprised of 20 studies found that variation within each study and the section of the gut sampled had the greatest effect on the microbiota (Holman, Brunelle, Trachsel, Allen, & Bik, 2017). These examples clearly demonstrate several implications when interpretating results from microbiome analyses, especially across different studies. Thus, the use of

microbiome analyses as a monitoring tool is challenging since the reproducibility and reliability are questioned today. In addition, the causal relationship between microbiome, host and environment remains unclear.

In terms of the actual sampling, the gill is compared to several other organs easily accessible on the fish. Tissue samples for histopathology are further preserved in formalin, a medium stored at room temperature which facilitates sample management. Therefore, the variation introduced due to sampling procedures would be considered limited using gill histology. It is, however, important to sample identical areas of the gill, since typically one gill arch (or part of it) constitutes the sample sent for analysis (Paper III). Mucous sampling with swabs for microbiome analyses is also readily applicable (Paper IV). However, contamination is a challenge when sampling for analyses targeting DNA material (Millar, Xu, & Moore, 2002). Hence, there is an increased risk of introducing variation from sampling procedures using methods including PCR technology compared to histopathology methods. One advantage of microbiome analyses of mucus from the surface of fish (e.g., gill or skin) is that sampling can be performed non-lethally with a swab (Slinger, Adams, & Wynne, 2021). Gill histology sampling and several other sampling routines require the fish to be sacrificed as part of sampling. Sampling of fish is extensive in salmon production today, and non-lethal sampling methods are thus appreciated.

Another aspect regarding reliability in studies comparing fish performance is differences between land-based production and marine farms. Assessed from farm visits, production parameters and building infrastructure, the RAS hatcheries included in Papers III-IV appeared very diverse. Traditional marine farms with open net cages share several infrastructure features and hence biological risk factors. Hatcheries are much more diverse in terms of their appearance (e.g. how they are built and function), and this is of importance when comparing fish from different hatcheries. Each RAS facility basically operates as independent ecosystems, partly due to differences in the building infrastructure (Dahle et al., 2020; Fossmark et al., 2021; Minich et al., 2020). When comparing fish performance between hatcheries, these differences must therefore be considered to a larger extent than in similar investigations between marine farms.

5.6 Conclusion and future perspective

The aim of this thesis was to investigate monitoring methods to improve fish health management in salmon farming. Fish health can be monitored using various methods and at different levels in the production hierarchy. In Paper I mortality patterns in salmon farming using cause-specific mortality classification (objective 1) was analysed and a standard for classification of cause-specific mortality in salmon aquaculture (objective 2) was suggested in Paper II. Findings from these studies showed that utilizing a cause-specific mortality classification method identifies causes and patterns of mortality during the production cycle. This method can be applied at any desired level of detail and level in the production hierarchy. This makes it a valuable tool which provides a substantial amount of information with limited effort and low cost. In addition, the proposed cause-specific mortality classification system developed in Paper II has been incorporated in the major production system databases in salmon farming (Fishtalk and Mercatus) and is included in the revised standard "Salmon and rainbow trout - Unambiguous terminology and methods for documentation of production" (NS 9417) by Standards Norway (personnel comment Olav Jamtøy). A unified method of cause specific mortality classification has a potential to aid monitoring and strategic diseases control at a regional and national level.

The third objective of this thesis was to explore whether gill mucous cell numbers and gill mucus microbial composition could be used as indicators of gill health status (objective 3) which was studied in Paper III-IV. The results in Paper III showed that mucous cell count varied both between the investigated fish and between farms. However, further studies are required to develop this into an early warning system to detect deteriorating health in the population. Paper IV suggested the individual fish as the most important source of variation in gill microbial diversity with sparse clustering on farm-level. This indicates that using microbial diversity as an indicator of fish health at population level will pose a challenge for now. Further studies are needed to untangle the relationship between the microbial composition of the host, health status of the host and the rearing environment to further investigate the potential as a monitoring tool of fish health.

The methods to assess and monitor health utilised in the included studies varied in both context and approach. Paper I-II used a measurement of health on population level, where a mortality cause was recorded for each dead fish in the population as a daily count attributed to a causal category. The method thus provides both

qualitative and quantitative information. The method can be used at all stages of the aquaculture production, exemplified in Paper I by studying the marine phase of salmon production. In Paper III-IV individual health measurements of each fish were used when mucous cell count and microbial diversity was investigated as potential outcome. The studies were performed in hatcheries on land. Since the environment in the land-based facilities can be controlled to some extent, monitoring health is important to react early upon changes in the environment and sustain a healthy fish. Microbial composition and mucous cell count further represent measurements that, if proven successful, would provide the industry with objective and scientifically reproducible information. However, they do measure the outcome on an individual level, meaning there is variation within the population. In comparison, mortality causes provide information solely on population level and includes information from all individuals in that population. Mucus cell count and gill microbial composition (Paper III-IV) will practically always be measured on a few individuals selected from the population. This selection introduces a bias regarding if those individuals are representative of the entire population of interest. This particular bias is not present in a population measurement such as mortality causes, however, there are other biases (for example subjectivity in assessment of the mortality cause of each fish).

The work with this thesis has also revealed the importance of the population unit in aquaculture (Papers I-IV), and sources of variation (Papers I and IV). Papers I-II clearly state the importance of the fish-group, primarily as identifier of the population unit at lowest level in aquaculture, but also where (unexplained) variation in mortality resides (Paper I). The latter is important since it indicates a need to improve monitoring on the fish-group level to further investigate causes of mortality. Paper IV points to the fish as the most important source of variation in microbial diversity, raising the question of causality in the relationship between the immune system of the fish and the microbiome. Managing production through the monitoring of microbial expression in gill mucous would be challenging if the immune system alters the microbiome. In general, causality and sources of variation are essential factors when considering monitoring methods for fish health. If causality is not established, intervention based on monitoring results would not improve health.

It is important to assess the cost-benefit of new health monitoring methods. Cause specific mortality is relatively easy to implement and the results can be evaluated directly. One drawback is the uncertainty when subjective assessment is used to assign the mortality cause. However, studies on the human system of mortality causes assess this bias as limited if the mortality derive from an event (e.g., car-crash). Since most mortalities in salmon production today is event-based, this bias can be assumed to be limited in aquaculture. The microbial composition and mucous cell count represents measuring methods that requires sacrifice of the fish, in addition to cost of analysing the samples. Such monitoring must be performed regularly, and sampling frequency will further increase the cost. According to the results in Paper III-IV, monitoring mucous cell count and gill microbial composition will yield limited benefits for the farmer today. On the contrary, cause specific mortality registration has a low cost of implementation and provides documented benefits for the farmer.

Suggestions for industry development and further studies:

- Improve tracking of fish-groups within production management systems.
- Systematise existing health data (e.g., smolt documentation sheets, reports from fish health visits, laboratory results etc) and implement the information into the production management systems
- Perform further studies to validate the proposed system of cause-specific mortality registration.
- Establish a baseline for gill mucous cell counts and perform longitudinal studies investigating the relation between the number of mucous cells, fish size and health status of the fish.
- Investigate relationship between microbial expression of different microbial ecosystems in fish production and interaction with the fish's immune system.

6 References

Akvakulturdriftsforskriften. (2008). Forskrift om drift av akvakulturanlegg. Retrieved from https://lovdata.no/forskrift/2008-06-17-822/

Arup, T. (2002). Transparency of the North Sea and Baltic Sea-a Secchi depth data mining study. *Oceanologia, 44*(3).

Atanasova, K. R., & Reznikov, L. R. (2019). Strategies for measuring airway mucus and mucins. *Respiratory Research, 20*(1), 261. doi:10.1186/s12931-019-1239-z

Aunsmo, A., Bruheim, T., Sandberg, M., Skjerve, E., Romstad, S., & Larssen, R. B. (2008). Methods for investigating patterns of mortality and quantifying cause-specific mortality in sea-farmed Atlantic salmon Salmo salar. *Diseases of Aquatic Organisms, 81*(2), 99-107. doi:10.3354/dao01954

Aunsmo, A., Valle, P. S., Sandberg, M., Midtlyng, P. J., & Bruheim, T. (2010). Stochastic modelling of direct costs of pancreas disease (PD) in Norwegian farmed Atlantic salmon (Salmo salar L.). *Preventive Veterinary Medicine, 93*(2-3), 233-241. doi:10.1016/j.prevetmed.2009.10.001

Awata, S., Tsuruta, T., Yada, T., & Iguchi, K. i. (2011). Effects of suspended sediment on cortisol levels in wild and cultured strains of ayu Plecoglossus altivelis. *Aquaculture, 314*(1), 115-121. doi:10.1016/j.aquaculture.2011.01.024

Bancroft, J. D., & Gamble, M. (2008). *Theory and Practice of Histological Techniques*. London: Churchill Livingstone.

Bang Jensen, B., Mårtensson, A., & Kristoffersen, A. B. (2020). Estimating risk factors for the daily risk of developing clinical cardiomyopathy syndrome (CMS) on a fishgroup level. *Preventive Veterinary Medicine, 175*, 104852. doi:10.1016/j.prevetmed.2019.104852

Bang Jensen, B., Qviller, L., & Toft, N. (2020). Spatio-temporal variations in mortality during the seawater production phase of Atlantic salmon (Salmo salar) in Norway. *Journal of Fish Diseases, 43*(4), 445-457. doi:10.1111/jfd.13142

Becke, C., Schumann, M., Steinhagen, D., Geist, J., & Brinker, A. (2018). Physiological consequences of chronic exposure of rainbow trout (Oncorhynchus mykiss) to suspended solid load in recirculating aquaculture systems. *Aquaculture, 484*, 228-241. doi:10.1016/j.aquaculture.2017.11.030

Benktander, J., Padra, J. T., Maynard, B., Birchenough, G., Botwright, N. A., McCulloch, R., . . . Lindén, S. K. (2020). Gill Mucus and Gill Mucin O-glycosylation in Healthy and Amebic Gill Disease-Affected Atlantic Salmon. *Microorganisms, 8*(12). doi:10.3390/microorganisms8121871

Berggren, H., Tibblin, P., Yıldırım, Y., Broman, E., Larsson, P., Lundin, D., & Forsman, A. (2022). Fish Skin Microbiomes Are Highly Variable Among Individuals and Populations but Not Within Individuals. *Frontiers in Microbiology, 12*. doi:10.3389/fmicb.2021.767770

Bjørgen, H., & Koppang, E. O. (2021). Anatomy of teleost fish immune structures and organs. *Immunogenetics, 73*(1), 53-63. doi:10.1007/s00251-020-01196-0

Blood, D. C., Studdert, V. P., & Gay, C. C. (2007). *Saunders Comprehensive Veterinary Dictionary.* (3 ed.). London: Elsevier Science.

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., . . . Caporaso, J. G. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology, 37*(8), 852-857. doi:10.1038/s41587-019-0209-9

Brambell, F. (1965). Report on the Technical Committee to enquire into the welfare of livestock kept under intensive conditions. *Her Majesty's Stationary Office, London*.

Brooke, H. L., Talbäck, M., Hörnblad, J., Johansson, L. A., Ludvigsson, J. F., Druid, H., . . . Ljung, R. (2017). The Swedish cause of death register. *European Journal of Epidemiology, 32*(9), 765-773. doi:10.1007/s10654-017-0316-1

Bruno, D., Noguera, P., & Poppe, T. (2013). *A Colour Atlas of Salmonid Diseases*. New York London: Springer Dordrecht Heidelberg

Bruton, M. N. (1985). The effects of suspensoids on fish. *Hydrobiologia, 125*(1), 221-241. doi:10.1007/BF00045937

Chen, Y., Nickola, T. J., DiFronzo, N. L., Colberg-Poley, A. M., & Rose, M. C. (2006). Dexamethasone-Mediated Repression of MUC5AC Gene Expression in Human Lung Epithelial Cells. *American journal of respiratory cell and molecular biology, 34*(3), 338-347. doi:10.1165/rcmb.2005-0176OC

Chen, S., Timmons, M. B., Aneshansley, D. J., & Bisogni, J. J. (1993). Suspended solids characteristics from recirculating aquacultural systems and design implications. *Aquaculture, 112*(2), 143-155. doi:doi.org/10.1016/0044-8486(93)90440-A

Clarke, E. A. (1974). What is Preventive Medicine? *Canadian family physician Medecin de famille canadien, 20*(11), 65-68.

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

Dalsgaard, J., Lund, I., Thorarinsdottir, R., Drengstig, A., Arvonen, K., & Pedersen, P. B. (2013). Farming different species in RAS in Nordic countries: Current status and future perspectives. *Aquacultural Engineering, 53*, 2-13. doi:doi.org/10.1016/j.aquaeng.2012.11.008

Dang, M., Pittman, K., Bach, L., Sonne, C., Hansson, S. V., Søndergaard, J., . . . Nowak, B. (2019). Mucous cell responses to contaminants and parasites in shorthorn sculpins (Myoxocephalus scorpius) from a former lead-zinc mine in West Greenland. *Science of The Total Environment, 678*, 207-216. doi:10.1016/j.scitotenv.2019.04.412

Davidson, J., Good, C., Williams, C., & Summerfelt, S. T. (2017). Evaluating the chronic effects of nitrate on the health and performance of post-smolt Atlantic salmon Salmo salar in freshwater recirculation aquaculture systems. *Aquacultural Engineering, 79*, 1-8. doi:10.1016/j.aquaeng.2017.08.003

de Jesus Gregersen, K. J., Pedersen, P. B., Pedersen, L.-F., & Dalsgaard, J. (2019). Micro particles and microbial activity in Danish recirculating rainbow trout (Oncorhynchus mykiss) farms. *Aquacultural Engineering, 84*, 60-66. doi:10.1016/j.aquaeng.2018.12.001

Directorate of fisheries. (2022a, 20 February). Biomassestatistikk etter produksjonsomraade. Retrieved from https://www.fiskeridir.no/Akvakultur/Tall-og-analyse/Biomassestatistikk/Biomassestatistikk-etter-produksjonsomraade

Directorate of Fisheries. (2022b, 20 February). Norwegian aquaculture statistics. Retrieved from https://www.fiskeridir.no/Akvakultur/Tall-og-analyse/Akvakulturstatistikk-tidsserier/Laks-regnbueoerret-og-oerret

Dohoo, I. R., Martin, S. W., & Stryhn, H. (2009). *Veterinary epidemiologic research*. Charlotte, P.E.I.: VER, Inc.

Dyrevelferdsloven. (2009). Lov om dyrevelferd. Retrieved from https://lovdata.no/lov/2009-06-19-97

Ellis, T., Berrill, I., Lines, J., Turnbull, J. F., & Knowles, T. G. (2012). Mortality and fish welfare. *Fish Physiology and Biochemistry, 38*(1), 189-199. doi:10.1007/s10695-011-9547-3

Ervik, L.-C., Larsen, J. S., Klakegg, B., Sandberg, M. G., Johansen, E., & Holmøy, R. (2020). *Smittesikring og biosikkerhet i norsk lakseproduksjon, delrapport 1 (in Norwegian: Infection controll and biosecurity in Norwegian salmon production, report 1)*. Retrieved from BDO and Åkerblå: https://www.fhf.no/prosjekter/prosjektbasen/901522/

Evans, D. H., Piermarini, P. M., & Choe, K. P. (2005). The Multifunctional Fish Gill: Dominant Site of Gas Exchange, Osmoregulation, Acid-Base Regulation, and Excretion of Nitrogenous Waste. *Physiological Reviews, 85*(1), 97-177. doi:10.1152/physrev.00050.2003

Ferguson, H. W. (2006). *Systemic Pathology of Fish: A Text and Atlas of Normal Tissues in Teleosts and Their Responses in Disease*. London: Scotian Press.

Ferguson, H. W., Morrison, D., Ostland, V. E., Lumsden, J., & Byrne, P. (1992). Responses of mucus-producing cells in gill disease of rainbow trout (Oncorhynchus mykiss). *Journal of Comparative Pathology, 106*(3), 255-265. doi:10.1016/0021-9975(92)90054-X

Fernandes, P. M., Pedersen, L.-F., & Pedersen, P. B. (2017). Influence of fixed and moving bed biofilters on micro particle dynamics in a recirculating aquaculture system. *Aquacultural Engineering, 78*, 32-41. doi:10.1016/j.aquaeng.2016.09.002

Folmar, L. C., & Dickhoff, W. W. (1980). The parr—Smolt transformation (smoltification) and seawater adaptation in salmonids: A review of selected literature. *Aquaculture, 21*(1), 1-37. doi:10.1016/0044-8486(80)90123-4

Forskrift om lakselusbekjempelse. (2013). Forskrift om bekjempelse av lakselus i akvakulturanlegg. Retrieved from https://lovdata.no/forskrift/2012-12-05-1140

Forskrift om sykdom hos dyr. (2015). Forskrift om varsel og melding om sjukdom hos dyr. Retrieved from https://lovdata.no/dokument/SF/forskrift/2014-12-19-1841

Forskrift om tiltak for å forebygge, begrense og bekjempe PD hos akvakulturdyr. (2017). Forskrift om tiltak for å forebygge, begrense og bekjempe pankreassykdom (PD) hos akvakulturdyr. Retrieved from https://lovdata.no/forskrift/2017-08-29-1318

Fossmark, R. O., Attramadal, K. J. K., Nordøy, K., Østerhus, S. W., & Vadstein, O. (2021). A comparison of two seawater adaptation strategies for Atlantic salmon post-smolt (Salmo salar) grown in recirculating aquaculture systems (RAS): Nitrification, water and gut microbiota, and performance of fish. *Aquaculture, 532*, 735973. doi:10.1016/j.aquaculture.2020.735973

Foyle, K. L., Hess, S., Powell, M. D., & Herbert, N. A. (2020). What Is Gill Health and What Is Its Role in Marine Finfish Aquaculture in the Face of a Changing Climate? *Frontiers in Marine Science, 7*(400). doi:10.3389/fmars.2020.00400

Francis-Floyd, R. (2005). *Introduction to fish health management*. Retrieved from Institute of food and agricultural sciences, University of Florida: http://ufdcimages.uflib.ufl.edu/IR/00/00/17/85/00001/FA00400.pdf

Friedrichs, K. R., Harr, K. E., Freeman, K. P., Szladovits, B., Walton, R. M., Barnhart, K. F., & Blanco-Chavez, J. (2012). ASVCP reference interval guidelines: determination of de novo reference intervals in veterinary species and other related topics. *Veterinary Clinical Pathology, 41*(4), 441-453. doi:10.1111/vcp.12006

Geitung, L., Oppedal, F., Stien, L. H., Dempster, T., Karlsbakk, E., Nola, V., & Wright, D. W. (2019). Snorkel sea-cage technology decreases salmon louse infestation by 75% in a full-cycle commercial test. *International Journal for Parasitology, 49*(11), 843-846. doi:10.1016/j.ijpara.2019.06.003

Gomez, D., Sunyer, J. O., & Salinas, I. (2013). The mucosal immune system of fish: The evolution of tolerating commensals while fighting pathogens. *Fish & Shellfish Immunology, 35*(6), 1729-1739. doi:10.1016/j.fsi.2013.09.032

Grefsrud, E., Glover, K., Grøsvik, B., Husa, V., Karlsen, Ø., Kristiansen, T., . . . Svåsand, T. (2018). *Risikorapport norsk fiskeoppdrett 2018*. Retrieved from https://www.hi.no/hi/nettrapporter/risikorapport-norsk-fiskeoppdrett/2018/risikorapport_2018

Gudding, R., & Van Muiswinkel, W. B. (2013). A history of fish vaccination: Science-based disease prevention in aquaculture. *Fish & Shellfish Immunology, 35*(6), 1683-1688. doi:10.1016/j.fsi.2013.09.031

Gunnarsson, S. (2006). The conceptualisation of health and disease in veterinary medicine. *Acta Veterinaria Scandinavica, 48*(1), 20. doi:10.1186/1751-0147-48-20

Guzman, K., Gray, T. E., Yoon, J. H., & Nettesheim, P. (1996). Quantitation of mucin RNA by PCR reveals induction of both MUC2 and MUC5AC mRNA levels by retinoids. *American Journal of Physiology, 271*(6 Pt 1), L1023-1028. doi:10.1152/ajplung.1996.271.6.L1023

Gåsnes, S. K., Oliveira, V. H. S., Gismervik, K., Ahimbisibwe, A., Tørud, B., & Jensen, B. B. (2021). Mortality patterns during the freshwater production phase of salmonids in Norway. *Journal of Fish Diseases, 44*(12), 2083-2096. doi:10.1111/jfd.13522

Haddeland, S., Lazado, C. C., Merkin, G. V., Myre, O. J., Okubamichael, M. A., Pedersen, L.-F., & Pittman, K. (2021). Dynamic morphometrics of mucous cells reveal the minimal impact of therapeutic doses of peracetic acid on Atlantic salmon gill health. *Aquaculture, 534*, 736315. doi:10.1016/j.aquaculture.2020.736315

Heier, B. T., Høgåsen, H. R., & Jarp, J. (2002). Factors associated with mortality in Norwegian broiler flocks. *Preventive Veterinary Medicine, 53*(1), 147-158. doi:10.1016/S0167-5877(01)00266-5

Hernández, B., Ramírez-Villalobos, D., Romero, M., Gómez, S., Atkinson, C., & Lozano, R. (2011). Assessing quality of medical death certification: Concordance between gold standard diagnosis and underlying cause of death in selected Mexican hospitals. *Population Health Metrics, 9*(1), 38. doi:10.1186/1478-7954-9-38

Hillestad, B., Johannessen, S., Melingen, G. O., & Moghadam, H. K. (2021). Identification of a New Infectious Pancreatic Necrosis Virus (IPNV) Variant in Atlantic Salmon (Salmo salar L.) that can Cause High Mortality Even in Genetically Resistant Fish. *Frontiers in Genetics, 12*. doi:10.3389/fgene.2021.635185

Holman, D. B., Brunelle, B. W., Trachsel, J., Allen, H. K., & Bik, H. (2017). Meta-analysis To Define a Core Microbiota in the Swine Gut. *mSystems, 2*(3), e00004-00017. doi:10.1128/mSystems.00004-17

Iversen, A,. Asche, F., Hermansen, Ø., & Nystøyl, R. (2020). Production cost and competitiveness in major salmon farming countries 2003–2018. *Aquaculture, 522*, 735089. doi:10.1016/j.aquaculture.2020.735089

Iversen, M., Finstad, B., McKinley, R. S., Eliassen, R. A., Carlsen, K. T., & Evjen, T. (2005). Stress responses in Atlantic salmon (Salmo salar L.) smolts during commercial well boat transports, and effects on survival after transfer to sea. *Aquaculture, 243*(1), 373-382. doi:10.1016/j.aquaculture.2004.10.019

Iversen, A,. Hermansen, Ø., Nystøyl, R., Hess, E. J., Rolland, K. H., Garshol, L. D., & Marthinussen, A. (2019). Kostnadsutvikling og forståelse av drivkrefter i norsk lakseoppdrett. Faglig sluttrapport. *Nofima rapportserie*.

Jadotte, Y. T., & Lane, D. S. (2021). Core functions, knowledge bases and essential services: A proposed prescription for the evolution of the preventive medicine specialty. *Preventive medicine, 143*, 106286-106286. doi:10.1016/j.ypmed.2020.106286

Johansson, L.-H., Timmerhaus, G., Afanasyev, S., Jørgensen, S. M., & Krasnov, A. (2016). Smoltification and seawater transfer of Atlantic salmon (Salmo salar L.) is associated with systemic repression of the immune transcriptome. *Fish & Shellfish Immunology, 58*, 33-41. doi:10.1016/j.fsi.2016.09.026

Karlsen, C., Ottem, K. F., Brevik Ø, J., Davey, M., Sørum, H., & Winther-Larsen, H. C. (2017). The environmental and host-associated bacterial microbiota of Arctic seawater-farmed Atlantic salmon with ulcerative disorders. *Journal of Fish Diseases, 40*(11), 1645-1663. doi:10.1111/jfd.12632

Kibenge, F. S. B., & Powell, M. D. (2020). *Aquaculture health management, design and operation approaches*. London: Academic Press.

Knauer, M. T., & Hostetler, C. E. (2013). US swine industry productivity analysis, 2005 to 2010. *Journal of Swine Health and Production, 21*(5), 248-252. Retrieved from https://www.aasv.org/shap/issues/v21n5/v21n5p248.html

Knibbs, G. H. (1929). The International Classification of Disease and Causes of Death and its Revision. *Medical Journal of Australia, 1*, 2-12. Retrieved from https://www.cabdirect.org/cabdirect/abstract/19292701569

Koppang, E. O., Kvellestad, A., & Fischer, U. (2015). Fish mucosal immunity: gill. In B. H. Beck & E. Peatman (Eds.), *Mucosal health in aquaculture* (pp. 94-120). London: Academic press.

Legrand, T. P. R. A., Wynne, J. W., Weyrich, L. S., & Oxley, A. P. A. (2020). A microbial sea of possibilities: current knowledge and prospects for an improved understanding of the fish microbiome. *Reviews in Aquaculture, 12*(2), 1101-1134. doi:10.1111/raq.12375

Lin, H., Carlson, D. M., St. George, J. A., Plopper, C. G., & Wu, R. (1989). An ELISA Method for the Quantitation of Tracheal Mucins from Human and Nonhuman Primates. *American journal of respiratory cell and molecular biology, 1*(1), 41-48. doi:10.1165/ajrcmb/1.1.41

Llewellyn, M. S., Boutin, S., Hoseinifar, S. H., & Derome, N. (2014). Teleost microbiomes: the state of the art in their characterization, manipulation and importance in aquaculture and fisheries. *Frontiers in Microbiology, 5*(207). doi:10.3389/fmicb.2014.00207

Lozano, R., Lopez, A. D., Atkinson, C., Naghavi, M., Flaxman, A. D., & Murray, C. J. L. (2011). Performance of physician-certified verbal autopsies: multisite validation study using clinical diagnostic gold standards. *Population Health Metrics, 9*(1), 32. doi:10.1186/1478-7954-9-32

Marchesi, J. R., & Ravel, J. (2015). The vocabulary of microbiome research: a proposal. *Microbiome, 3*(1), 31. doi:10.1186/s40168-015-0094-5

Marcos-López, M., Calduch-Giner, J. A., Mirimin, L., MacCarthy, E., Rodger, H. D., O'Connor, I., . . . Piazzon, M. C. (2018). Gene expression analysis of Atlantic salmon gills reveals mucin 5 and interleukin 4/13 as key molecules during amoebic gill disease. *Scientific Reports, 8*(1), 13689. doi:10.1038/s41598-018-32019-8

Mardones, F. O. (2020). 6 - Preventive medicine of aquatic animals. In F. S. B. Kibenge & M. D. Powell (Eds.), *Aquaculture Health Management* (pp. 163-186): Academic Press.

McCormick, S. D. (2012). Smolt Physiology and Endocrinology. In S. D. McCormick, A. P. Farrell, & C. J. Brauner (Eds.), *Euryhaline Fishes* (Vol. 32, pp. 199-251): Academic Press.

Millar, B. C., Xu, J., & Moore, J. E. (2002). Risk assessment models and contamination management: implications for broad-range ribosomal DNA PCR as a diagnostic tool in medical bacteriology. *Journal of clinical microbiology, 40*(5), 1575-1580. doi:10.1128/JCM.40.5.1575-1580.2002

Minich, J. J., Poore, G. D., Jantawongsri, K., Johnston, C., Bowie, K., Bowman, J., . . . Allen, E. E. (2020). Microbial Ecology of Atlantic Salmon (Salmo salar) Hatcheries: Impacts of the Built Environment on Fish Mucosal Microbiota. *Applied and Environmental Microbiology, 86*(12). doi:10.1128/aem.00411-20

Moen, T., Baranski, M., Sonesson, A. K., & Kjøglum, S. (2009). Confirmation and fine-mapping of a major QTL for resistance to infectious pancreatic necrosis in Atlantic salmon (Salmo salar): population-level associations between markers and trait. *BMC Genomics, 10*(1), 368. doi:10.1186/1471-2164-10-368

Murray, C. J. L., Lozano, R., Flaxman, A. D., Vahdatpour, A., & Lopez, A. D. (2011). Robust metrics for assessing the performance of different verbal autopsy cause assignment methods in validation studies. *Population Health Metrics, 9*(1), 28. doi:10.1186/1478-7954-9-28

Naghavi, M., Abajobir, A. A., Abbafati, C., Abbas, K. M., Abd-Allah, F., Abera, S. F., . . . Murray, C. J. L. (2017). Global, regional, and national age-sex specific mortality for 264 causes of death, 1980–2016: a systematic analysis for the Global Burden of Disease Study 2016. *The Lancet, 390*(10100), 1151-1210. doi:10.1016/S0140-6736(17)32152-9

Nilsen, A., Nielsen, K. V., & Bergheim, A. (2020). A closer look at closed cages: Growth and mortality rates during production of post-smolt Atlantic salmon in marine closed confinement systems. *Aquacultural Engineering, 91*, 102124. doi:10.1016/j.aquaeng.2020.102124

Noble, C., Gismervik, K., Iversen, M. H., Kolarevic, J., Nilsson, J., Stien, L. H., & Turnbull, J. F. (2018). *Welfare Indicators for farmed Atlantic salmon: Tools for assessing fish welfare*. Retrieved from NOFIMA: http://www.nofima.no/fishwell/english

Næve, I., Korsvoll, S. A., Santi, N., Medina, M., & Aunsmo, A. (2022). The power of genetics: Past and future contribution of balanced genetic selection to sustainable growth and productivity of the Norwegian Atlantic salmon (Salmo salar) industry. *Aquaculture*, 738061. doi:10.1016/j.aquaculture.2022.738061

Oliveira, V. H. S., Dean, K. R., Qviller, L., Kirkeby, C., & Bang Jensen, B. (2021). Factors associated with baseline mortality in Norwegian Atlantic salmon farming. *Scientific Reports, 11*(1), 14702. doi:10.1038/s41598-021-93874-6

Overton, K., Dempster, T., Oppedal, F., Kristiansen, T. S., Gismervik, K., & Stien, L. H. (2019). Salmon lice treatments and salmon mortality in Norwegian aquaculture: a review. *Reviews in Aquaculture, 11*(4), 1398-1417. doi:10.1111/raq.12299

Pettersen, J. M., Osmundsen, T., Aunsmo, A., Mardones, F. O., & Rich, K. M. (2015). Controlling emerging infectious diseases in salmon aquaculture. *Revue scientifique et technique, 34*(3), 923-938. doi:10.20506/rst.34.3.2406

Pettersen, J. M., Rich, K. M., Jensen, B. B., & Aunsmo, A. (2015). The economic benefits of disease triggered early harvest: A case study of pancreas disease in farmed Atlantic salmon from Norway. *Preventive Veterinary Medicine, 121*(3-4), 314-324. doi:10.1016/j.prevetmed.2015.08.003

Pittman, K., Pittman, A., Karlson, S., Cieplinska, T., Sourd, P., Redmond, K., . . . Sweetman, E. (2013). Body site matters: an evaluation and application of a novel histological methodology on the quantification of mucous cells in the skin of Atlantic salmon, Salmo salar L. *Journal of Fish Diseases, 36*(2), 115-127. doi:10.1111/jfd.12002

Pittman, K., Sourd, P., Ravnoy, B., Espeland, O., Fiksdal, I. U., Oen, T., . . . Sweetman, J. (2011). Novel method for quantifying salmonid mucous cells. *Journal of Fish Diseases, 34*(12), 931-936. doi:10.1111/j.1365-2761.2011.01308.x

Poppe, T., & Bergh, Ø. (1999). *Fiskehelse og fiskesykdommer*: Universitetsforlaget.

Quigley, M. A., Chandramohan, D., & Rodrigues, L. C. (1999). Diagnostic accuracy of physician review, expert algorithms and data-derived algorithms in adult verbal autopsies. *International Journal of Epidemiology, 28*(6), 1081-1087. doi:10.1093/ije/28.6.1081

Roberts, S. D., & Powell, M. D. (2003). Comparative ionic flux and gill mucous cell histochemistry: effects of salinity and disease status in Atlantic salmon (Salmo salar L.). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 134*(3), 525-537. doi:10.1016/S1095-6433(02)00327-6

Rodger, H. D. (2007). Gill disorders: an emerging problem for farmed Atlantic salmon (Salmo salar) in the marine environment? *Fish Veterinary Journal*(9), 38-48.

Rodger, H. D., Henry, L., & Mitchell, S. O. (2011). Non-infectious gill disorders of marine salmonid fish. *Reviews in Fish Biology and Fisheries, 21*(3), 423-440. doi:10.1007/s11160-010-9182-6

Rose, G. (1981). Strategy of prevention: lessons from cardiovascular disease. *British medical journal (Clinical research ed.), 282*(6279), 1847-1851. doi:10.1136/bmj.282.6279.1847

Rosendal, G. K., & Olesen, I. (2022). Overcoming barriers to breeding for increased lice resistance in farmed Atlantic salmon: A case study from Norway. *Aquaculture, 548*, 737574. doi:10.1016/j.aquaculture.2021.737574

Salama, N. K. G., Murray, A. G., Christie, A. J., & Wallace, I. S. (2016). Using fish mortality data to assess reporting thresholds as a tool for detection of potential disease concerns in the Scottish farmed salmon industry. *Aquaculture, 450*, 283-288. doi:10.1016/j.aquaculture.2015.07.023

Schneider, E. C. (2002). Measuring Mortality Outcomes to Improve Health Care: Rational Use of Ratings and Rankings. *Medical Care, 40*(1), 1-3. doi:10.1097/00005650-200201000-00001

Segner, H., Sundh, H., Buchmann, K., Douxfils, J., Sundell, K. S., Mathieu, C., . . . Vaughan, L. (2012). Health of farmed fish: its relation to fish welfare and its utility as welfare indicator. *Fish Physiology and Biochemistry, 38*(1), 85-105. doi:10.1007/s10695-011-9517-9

Shephard, K. L. (1994). Functions for fish mucus. *Reviews in Fish Biology and Fisheries, 4*(4), 401-429. doi:10.1007/BF00042888

Shepherd, C. J., & Poupard, C. W. (1975). Veterinary aspects of salmonid fish farming: husbandry diseases. *Veterinary Record, 97*(3), 45-47. doi:10.1136/vr.97.3.45

Sinha, R., Abu-Ali, G., Vogtmann, E., Fodor, A. A., Ren, B., Amir, A., . . . The Microbiome Quality Control Project, C. (2017). Assessment of variation in microbial community amplicon sequencing by the Microbiome Quality Control (MBQC) project consortium. *Nature Biotechnology, 35*(11), 1077-1086. doi:10.1038/nbt.3981

Slinger, J., Adams, M. B., & Wynne, J. W. (2021). Comparison of bacterial diversity and distribution on the gills of Atlantic salmon (Salmo salar L.): an evaluation of sampling techniques. *Journal of Applied Microbiology, 131*(1), 80-92. doi:10.1111/jam.14969

Snieszko, S. F. (1974). The effects of environmental stress on outbreaks of infectious diseases of fishes. *Journal of Fish Biology, 6*(2), 197-208. doi:10.1111/j.1095-8649.1974.tb04537.x

Soares, S., Green, D. M., Turnbull, J. F., Crumlish, M., & Murray, A. G. (2011). A baseline method for benchmarking mortality losses in Atlantic salmon (Salmo salar) production. *Aquaculture, 314*(1), 7-12. doi:10.1016/j.aquaculture.2011.01.029

Soleman, N., Chandramohan, D., & Shibuya, K. (2006). Verbal autopsy: current practices and challenges. *Bulletin of the World Health Organization, 84*(3), 239-245. doi:10.2471/blt.05.027003

Sommerset, I., Bang Jensen, B., Bornø, B., Haukaas, A., & Brun, E. (2021). *Fish Health Report 2020*. Retrieved from Norwegian Veterinary Institute: https://www.vetinst.no/en/reports-and-publications/reports

Sommerset, I., Walde, C. S., Bang, J. B., Wiik-Nielsen, J., Bornø, G., Oliveira, V., . . . Brun, E. (2022). *Fiskehelserapporten 2021*. Retrieved from Norwegian Veterinary Institute: https://www.vetinst.no/rapporter-og-publikasjoner/rapporter/2022/fiskehelserapporten-2021

Speare, D. J., Arsenault, G., MacNair, N., & Powell, M. D. (1997). Branchial lesions associated with intermittent formalin bath treatment of Atlantic salmon, Salmo salar L., and rainbow trout, Oncorhynchus mykiss (Walbaum). *Journal of Fish Diseases, 20*(1), 27-33. doi:10.1046/j.1365-2761.1997.d01-103.x

Stien, L. H., Lind, M. B., Oppedal, F., Wright, D. W., & Seternes, T. (2018). Skirts on salmon production cages reduced salmon lice infestations without affecting fish welfare. *Aquaculture, 490*, 281-287. doi:10.1016/j.aquaculture.2018.02.045

Striberny, A., Lauritzen, D. E., Fuentes, J., Campinho, M. A., Gaetano, P., Duarte, V., . . . Jørgensen, E. H. (2021). More than one way to smoltify a salmon? Effects of dietary and light treatment on smolt development and seawater growth performance in Atlantic salmon. *Aquaculture, 532*, 736044. doi:10.1016/j.aquaculture.2020.736044

Sviland Walde, C., Bang Jensen, B., Pettersen, J. M., & Stormoen, M. (2021). Estimating cage-level mortality distributions following different delousing treatments of Atlantic salmon (salmo salar) in Norway. *Journal of Fish Diseases, 44*(7). doi:10.1111/jfd.13348

Tartor, H., Luis Monjane, A., & Grove, S. (2020). Quantification of Defensive Proteins in Skin Mucus of Atlantic salmon Using Minimally Invasive Sampling and High-Sensitivity ELISA. *Animals (Basel), 10*(8). doi:10.3390/ani10081374

Thrusfield, M., & Christley, R. (2018). *Veterinary epidemiology* (4th ed. ed.). Hoboken N.J: Wiley Blacwell.

Tlusty, M. F. (2020). 1 - Animal health: the foundation for aquaculture sustainability. In F. S. B. Kibenge & M. D. Powell (Eds.), *Aquaculture Health Management* (pp. 1-15): Academic Press.

Toft, N., Agger, F. J., Houe, H., & Bruun, J. (2004). Measures of disese frequency. In H. Houe, A. Kjaer Ersboll, & N. Toft (Eds.), *Introduction to veterinary epidemiology* (pp. 77-94). Frederiksberg: Biofolia.

Tyler, J. E. (1968). The secchi disc. *Limnology and Oceanography, 13*(1), 1-6. doi:10.4319/lo.1968.13.1.0001

Uren Webster, Rodriguez-Barreto, D., Castaldo, G., Gough, P., Consuegra, S., & Garcia de Leaniz, C. (2020). Environmental plasticity and colonisation history in the Atlantic salmon microbiome: A translocation experiment. *Molecular ecology, 29*(5), 886-898. doi:10.1111/mec.15369

van Rijn, J. (2013). Waste treatment in recirculating aquaculture systems. *Aquacultural Engineering, 53*, 49-56. doi:10.1016/j.aquaeng.2012.11.010

Verschuuren, M., & van Oers, H. (2019). *Population Health Monitoring. Climbing the Information Pyramid. 1st ed*. Cham: Springer Nature Switzerland.

World Health Organization. (1979). *Medical certification of cause of death, Instructions for physicians on use of international form of medical certificate of cause of death*. Retrieved from World Health Organization: https://apps.who.int/iris/handle/10665/40557

World Health Organization. (2011). *Review of the WHO Verbal Autopsy (VA) Instruments, Meeting Report*. Retrieved from World Health Organization: https://www.who.int/publications/m/item/review-of-the-who-verbal-autopsy-(va)-instruments

World Health Organization. (2016). *Verbal autopsy standards: The 2016 WHO verbal autopsy instrument (V.1.5.3)*. Retrieved from World Health Organization: https://www.who.int/publications/m/item/verbal-autopsy-standards-the-2016-who-verbal-autopsy-instrument

World Health Organization. (2019). *ICD-11, International classification of diseases for mortality and morbidity statistics*(11th Revision ed.). Retrieved from https://icd.who.int/en

World Health Organization. (2021a). *History of the development of the ICD*. Retrieved from World Health Organization: https://www.who.int/publications/m/item/history-of-the-development-of-the-icd

World Health Organization. (2021b). *World health statistics 2021: monitoring health for the SDGs, sustainable development goals*. Retrieved from World Health Organization: https://apps.who.int/iris/handle/10665/342703.

World Health Organization. (2022, 22 January). WHO constitution. Retrieved from https://www.who.int/about/governance/constitution

Zuhdi Alimam, M., Piazza, F. M., Selby, D. M., Letwin, N., Huang, L., & Rose, M. C. (2000). Muc-5/5ac Mucin Messenger RNA and Protein Expression Is a Marker of Goblet Cell Metaplasia in Murine Airways. *American journal of respiratory cell and molecular biology, 22*(3), 253-260. doi:10.1165/ajrcmb.22.3.3768

Østerås, O., Solbu, H., Refsdal, A. O., Roalkvam, T., Filseth, O., & Minsaas, A. (2007). Results and Evaluation of Thirty Years of Health Recordings in the Norwegian Dairy Cattle Population. *Journal of Dairy Science, 90*(9), 4483-4497. doi:10.3168/jds.2007-0030

7 Papers I-IV

Paper I

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RESEARCH ARTICLE

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Analysing mortality patterns in salmon farming using daily cage registrations

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Abstract

This study describes the patterns of mortality and investigates the sources of variation in mortality during the marine phase of commercial salmon farming. The study included daily mortality records from stocking to harvest of 21 million salmon from ten hatcheries in 136 fish-groups (fish in the same cage from the same hatchery). The fish was stocked in 2017–2018 at 21 marine farms within two Norwegian companies. The sources of variation in mortality were investigated using multilevel linear regression models with 'fish-group' nested within 'farm' as a random effect, cross-classified with 'hatchery'. In the final model, 'fish-group' was the source of most variation (70%). Furthermore, the mortality categories 'smolt-related mortality', 'infectious diseases' and 'handling and treatment' were responsible for 10%, 17% and 29% of the total number of dead fish respectively. Overall, the study shows that smolt-related mortality is one of the major causes of death in the first part of the production, while handling and treatment was the dominating cause of mortality in total. Mortality varied by fish-group to a large extent. This means that targeted preventive strategies to decrease mortality for individual fish-groups might be more effective than overall measures at farm or hatchery level.

KEYWORDS

cause-specific mortality, cross-classified multilevel regression model, fish health management, salmon farming, sources of variation

1 | INTRODUCTION

The life of a farmed salmon (*Salmo salar*) in Norway starts in the hatcheries, where the fish traditionally are hatched and raised to smolt in freshwater over a period of 6–12 months. The smolts are then transferred from the hatcheries into marine farms (marine phase), where they grow until harvested after another 12–18 months. Mortality during the production cycle will cause economic loss for the farmer but also has a cost in terms of reduced health and welfare for the fish. To prevent fish from dying, the causes and risk factors of mortality need to be investigated as a part of the fish health management at the farm. Analysis of registered biological data from the production is crucial when making decisions aimed at reducing mortality and subsequently improving fish welfare.

Cause-specific mortality is routinely recorded in most Norwegian salmon farms where the farm staffs assign a cause of death to each fish every day. The causal categories can be based on the macroscopic assessment of the fish, knowledge of events likely to have caused the mortality at the farm the last day (lice treatment, handling of fish, other operations at the farm, etc.) or information from fish health personnel after clinical investigation, autopsy or samples analysed. However, the practice of classifying cause-specific mortality

registration is currently not standardized, neither with a common list of causal categories nor with a common standard operating procedure. Cause-specific mortality is therefore not to be confused with a diagnosis, which is strictly given by fish health personnel.

The cause-specific mortality adds a qualitative attribute to the registrations compared with the sole crude mortality. Hence, the mortality data become both quantitative and qualitative and have a potential to help the farmer in health management and decision-making (Aunsmo et al., 2008; Nilsen et al., 2020).

Fish farmers report monthly mortality numbers from each farm to the Norwegian authorities, in accordance with national regulation (Akvakultudriftsforskriften, 2008). According to the 'fish health report' 2020, issued by the Norwegian Veterinary Institute, the median mortality of finished production cycles (fish transferred to sea at the same time to the same farm) at marine salmon farms in Norway 2020 was 17.9% (Sommerset et al., 2021). However, there is substantial variation in mortality between farms and production cycles, and mortality of more than 50% in single production cycles has been reported (Bang Jensen et al., 2020). The hatcheries also report mortality numbers to the authorities monthly. However, the information about hatchery mortality at a national level is scarce, since the reported numbers do not follow the fish throughout the production (Tørud et al., 2019). In addition, few standardized health measurements are performed when the salmon are transferred to sea from the hatcheries, and data on mortality causes at this stage are sparse at the national level. Hence, there are several knowledge gaps about the status of the smolt transferred to sea, mortality causes at both hatcheries and at sea, as well as how the early life of the salmon at the hatchery affects the later performance and survival in the marine phase (Bang Jensen et al., 2020; Gåsnes et al., 2021; Tørud et al., 2019).

Smoltification is a complex biological process where the physiology of the salmon transforms from life in a fresh water environment to saltwater (McCormick, 2012). This energy-demanding physiological transformation of the fish also affects the immune system negatively, making fish more vulnerable to stressful events and diseases during this period in life (Johansson et al., 2016). In salmon farming, the smoltification process is immediately followed by the transfer from hatchery to sea for the fish. The transfer involves several stressful events, including transportation, introduction to the saltwater environment and new pathogens (Iversen et al., 2005). These factors contribute to an increased risk of fish dying at the start of the sea phase. Bang Jensen, Qviller, et al. (2020) showed that there was an increase in mortality at the start of the sea phase compared with other periods in the production in Norway. Similar findings were also reported by Soares et al. (2011) from salmon production in Scotland. However, there is still limited information about the causes of 'smolt-related mortality' and what proportion of the overall mortality at cage level it constitutes in the marine phase.

Today, few scientific studies make use of production data retrieved directly from the production management systems (Fishtalk©, Akvagroup or Mercatus©, Scaleaqua) of the fish farmer (Bang Jensen, Mårtensson, et al., 2020). These production management systems were primarily built to help the farmer keep control of the inventory, i.e. number of fish, feeding and mortality. These are the primary tools used by farmers for following fish throughout production and hence constitute the most detailed records of daily mortality from each production unit. However, since the systems were not built as a fish health management tool primarily, the data need to be adapted when used to study fish health challenges. Such secondary use of data is common in veterinary epidemiological research and is considered a cost-effective way to perform population studies as long as the appropriateness of the data are assessed for the intended use (Houe et al., 2011; Sørensen et al., 1996).

The aim of this study was to investigate mortality patterns in the marine phase, both early (during the first 180 days) and for the entire production. This was approached through two objectives: To describe mortality during production using cause-specific mortality classifications from production management systems, and to estimate the variance component proportion (VCP) of mortality at different organizational levels in salmon production.

2 | MATERIAL AND METHODS

2.1 | Descriptive statistics

The study unit was the 'fish-group', defined as fish from the same hatchery transferred to sea at the same time and to the same cage. The fish was followed retrospectively through the entire marine phase from the day of transfer to sea ('day 0').

The study population consisted of 20,716,314 salmon in 136 cages at 21 marine farms belonging to two salmon farming companies. The fish was transferred to sea from ten land-based hatcheries in four consecutive stocking periods between spring 2017 and autumn 2018.

2.2 | Data sources and data management

Data for this study were collected from two sources. Data were extracted from the production management database of the farmers (Fishtalk©, Akvagroup) and from the documentation that followed the fish to the sea farm ('smolt documentation sheet').

Daily registrations on cage level were extracted in March 2020; Table 1 describes the details of the variables. In addition, a graphic timeline from the production system with an overview of all movements of the fish between cages in the farms was retrieved to help trace the fish-groups.

Information about hatching date and temperature at smolt farm (at the day of transfer to sea) was gathered from the 'smolt documentation sheet'. If absent, the information was collected through direct contact with the site manager at the smolt production site.

2.2.1 | Data management

Data from Fishtalk© were extracted as an Excel file (Microsoft Corporation), using a template made in Fishtalk© to ensure that

TABLE 1 Descriptive statistics of variables in the study, presented for each of the subsets I and II. Continuous variables are presented with mean, min and max values. Categorical variables are displayed with the number of fish-groups in each category. For the variables 'number of hatcheries with FT' and 'number of self-owned hatcheries', the associated number of fish-groups is shown in addition to the number of hatcheries

Variable	Subset I 'Early mortality'	Subset II 'Harvest mortality'
Number of fish-groups	121	74
Mortality in production [%], mean (min–max)	2.7 (0.3–21.2)	8.1 (2.7–23.9)
Ln-transformed outcome, mean (min–max)	0.58 (−1.17–3.05)	1.98 (0.99–3.18)
Number of hatcheries	10	9
Numbers of farms	20	16
Number of companies	2	2
Stocking period[a]		
Spring [number of fish-groups]	57	39
Autumn [number of fish-groups]	64	35
Year when transferred to sea		
2017 [number of fish-groups]	58	43
2018 [number of fish-groups]	63	31
Number of hatcheries with FT	7	7
Fish-group from hatcheries with FT	111	71
Number of self-owned hatcheries[a]	4	4
Fish-groups from self-owned hatcheries	90	55
Days in hatchery, mean (min–max)	350 (219–574)	361 (247–530)
Weight at transfer to sea [g], mean (min–max)	124 (74–250)	131 (74.3–250)
Temperature in sea at transfer [°C], mean (min–max)[a]	10.3 (4.9–16.0)	10 (5.0–16.0)
Delta temperature [°C], mean (min–max)[b]	0.8 (−5.3 – 4.5)	1.1 (−5.25–4.5)
Fish-groups treated against lice		
Yes [number of fish-groups]	7	60
No [number of fish-groups]	114	14
Fish-groups with 0 treatments against lice	–	14
Fish-groups with 1–4 treatments against lice[b]	–	42
Fish-groups with >4 treatments against lice[b]	–	18
Moved between cages		
Yes [number of fish-groups]	–	30
No [number of fish-groups]	–	44
Days in production, mean (min–max)	–	433 (352–517)

[a]Variable included in final model of 'early mortality'.
[b]Variable included in final model of 'harvest mortality'.

identical production information was retrieved from both companies. The information from the 'smolt documentation sheets' was plotted in an Excel file. All Excel files were imported to Stata (Stata SE/15; Stata Corp.) for further data management and statistical analysis.

One data set with two subsets were used in the study. The 'full data set' included all fish-groups transferred to sea and the registered daily mortality throughout the production. Two subsets of the 'full data set' were constructed in order to investigate the effect of smolt-related factors on cumulative mortality during the early marine phase (180 days post transfer, 'early mortality') and up until harvest ('harvest mortality') respectively. Fish-groups that had been either mixed, split or terminated were excluded. Five fish-groups that had not been harvested at the time of data extraction were also excluded. Only fish-groups that were traceable as one unit until the timepoint of interest was kept for the analysis, this being either during the first 180 days (subset I, early mortality) or up until harvest (subset II, harvest mortality; Figure 1).

2.3 | Mortality and cause-specific mortality

The two companies recorded cause-specific mortality daily. The data set contained a total of 65 different categories of mortality causes, including one for unknown cause. Several were also pointing towards the same cause, but with different names. Mortality causes associated with either 'smolt-related mortality', 'handling or lice treatment' or 'infectious diseases' were grouped together in three different groups targeting main challenges in Norwegian salmon

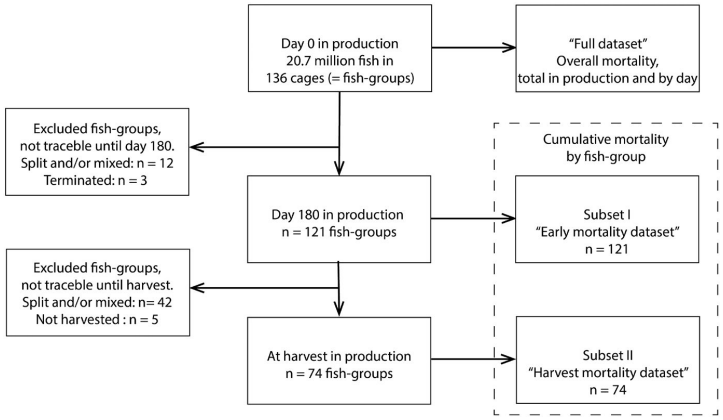

FIGURE 1 Flow chart describing the study population in the 'full data set' and the number of fish-groups in the two subsets 'early mortality' and 'harvest mortality'. Mortality measurements used are also indicated for each data set

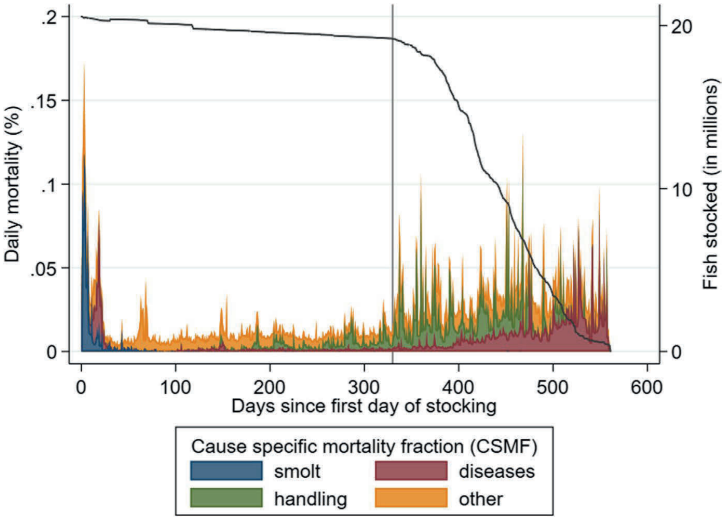

FIGURE 2 Graphic display of the daily mortality (%, left *y*-axis) from day of transfer to harvest in the 'full data set'. Coloured areas correspond to the different cause-specific mortality fractions (CSMF) within the mortality each day. The black line indicates the total number of fish in stock each day (right *y*-axis). The grey vertical line at day 330 is the first day with slaughter, meaning the population is, in addition to mortality, also decreasing because of harvest from this day and onwards

production today (Sommerset et al., 2021). The groups were named: 'smolt', 'handling' and 'diseases' respectively. All other causes were grouped in a fourth group 'other'. The group of 'smolt' included all mortality causes assessed to be related to the transition of fish from hatchery to the marine farm. Therefore, the mortality causes 'dead at arrival' and 'transportation', which indicate handling of fish, were included in the group of 'smolt' after a graphical assessment to when in the production mortality was recorded. Causes related to gill damages or gill diseases were first grouped in a separate group, but because of the low prevalence, they were later allocated to the group 'other'. An overview of all mortality classification categories with the grouping used for analysis is shown in Appendix 1.

Mortality in the full dataset was described as either 'daily mortality' (%) or 'total mortality' (%) across all fish-groups in the data set. 'Daily mortality' was the sum of dead fish each day divided by the total number of fish in all cages each day (Figure 2). 'Total mortality' was the sum of all dead fish, from transfer to harvest, divided by the sum of all fish transferred to sea. Mortality in the subsets was calculated for each fish-group. Cumulated mortality, at 180 days ('early mortality', %) or at harvest ('harvest mortality', %), was divided by the number of fish transferred to sea in each fish-group (Figures 1 and 3). The proportion in different mortality causes were described as cause-specific mortality fractions (CSMF, %), meaning the different causes of mortality were expressed as proportions of either the total number of dead fish or of the mortality expressions above (Figures 2 and 3).

2.4 | Variables and statistical analyses

2.4.1 | Variables

The exposures of interest for the statistical analyses were factors related to the transition of salmon from the hatchery to the sea site. This represents events in the production preceding the first period

in sea, which is identified as the time in production with highest risk of dying according to the earlier studies (Bang Jensen, Qviller, et al., 2020; Soares et al., 2011). In addition to those smolt-related variables, factors related to lice treatments and handling of fish were also included as this represents events known to cause extensive mortality throughout the production in sea (Sommerset et al., 2021). Variables tested in each model are shown in Table 1.

Eight variables associated with the sea transfer were included in the analysis (Table 1). Three were calculated from the raw data; 'days at smolt supplier' was a continuous variable based on the number of days from hatching to transfer to sea. The 'delta temperature' was a continuous variable based on the difference between the water temperature from the last day in the smolt facility to the daily average sea temperature at the sea farm the first week. 'Stocking period' was a dichotomous variable indicating whether the fish were transferred to sea within the first or last six months of the year. The other variables associated with sea transfer were as follows: fish weight at sea transfer ('weight at transfer to sea', continuous), whether the hatchery had flow through water system (FT); recirculation aquaculture systems or a combination of those, the two latter combined due to small numbers ('FT hatchery', dichotomous), if the hatchery was owned by the owner of the sea site or not ('self-owned', dichotomous); sea temperature at transfer, calculated as the daily average during the first week for each group as stated earlier ('temperature in sea at transfer', continuous) and hatchery ('hatchery').

To control for lice treatments and handling during the marine phase, in addition to other events that potentially affected the outcomes, seven other variables were included in the analyses. To account for salmon lice treatments, two treatment variables were constructed. The first indicated whether the fish-group had been treated against sea lice ('treated', dichotomous) and the second quantified the total number of treatments, which were categorized into three groups (0, 1–4 and >4 treatments) and named 'number of treatments' (categorical). The company and farm at sea ('company' and 'farm', categorical), which year the fish were transferred to sea ('year', categorical) and if the fish-group was moved to another cage during the marine phase of production ('moved', dichotomous). The number of days in production at sea ('days in production', continuous) was the number of days from transfer to sea until harvest (slaughter). For fish-groups harvested over multiple days, the 'days in production' was calculated as an average between the first and last day harvest.

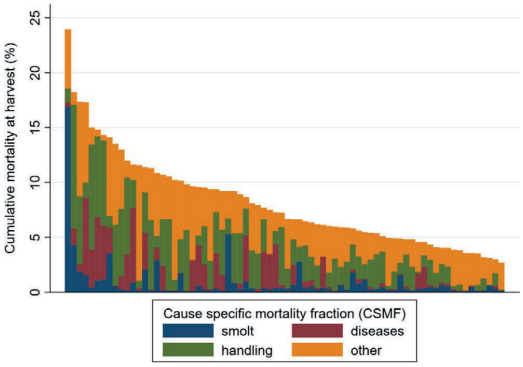

FIGURE 3 Graphical display of the cumulative mortality in each fish-group at harvest (subset II, $n = 74$ fish-groups), where the mortality is expressed as a proportion (%) of the fish transferred to sea. The different cause-specific mortality fractions (CSMF) are indicated for each fish-group with colours, and the fish-groups are sorted descending according to the total mortality

2.4.2 | Regression modelling

The outcome variables ('early mortality' and 'harvest mortality') had to be ln-transformed to reach the assumption of normally distributed residuals. All explanatory variables were tested with univariable linear regression for each of the two outcomes. Variables associated with the outcome at a level of $p < .1$ were included in further analyses. Models were built as multi level cross-classified linear regression models, using 'farm' as level and cross-classified with 'hatchery' (Figure 4). For the cross-classification multilevel modelling, MLwiN (MLwiN Version 3.05, University of Bristol) was used within Stata with the stata command 'runmlwin' (Charlton et al., 2020; Leckie & Charlton, 2012). The approach when building the model followed the method described by Aunsmo et al. (2009). Briefly, the modelling was performed using Markov chain Monte Carlo estimation with Gibbs sampling for the posterior distribution (Browne, 2019) with a burn-in period of 1500 iterations and a final model run of 100,000 iterations. To establish the prior

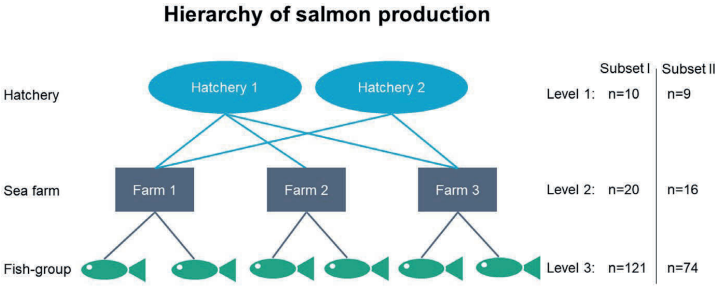

FIGURE 4 Illustration of the hierarchy of salmon production and the corresponding levels used in the cross-classified multilevel regression model for each of the subsets I ('early mortality') and II ('harvest mortality'). The number of units in each level for the two subsets is also indicated

distribution, the models were first run in iterative generalized least square. Models were built using forward selection and guided by a causal diagram. The improvement between models (for the same dependent variable) was evaluated using Bayesian deviance information criterion. Raftery-Lewis diagnostic and Brooks-Draper diagnostic were used as suggestion of the number of iterations, and the convergence of the models was assessed by kernel density plots (normality of the posterior estimates), plots for autocorrelation (AFC) and partial autocorrelation (PACF) (Aunsmo et al., 2009; Browne, 2019). The independent variables included in the final models were checked for collinearity using graphical assessments for the relationship between categorical and continuous variables. Residuals and trajectory plots were assessed for the final model for each outcome.

Sources of variation were investigated by comparing the proportion of the total variance explained by each random effect in the different models (Browne, 2019; Dohoo et al., 2001). The VCP was estimated for each level in the hierarchy for both the random intercept model and the final model. In addition, the variance component reduction (VCR) was estimated for each random effect, and overall, between the intercept model and the final model for each outcome (Aunsmo et al., 2009).

3 | RESULTS

3.1 | Descriptive statistics

The total mortality count of the full data set was 1,797,467 salmon, corresponding to an overall total mortality proportion of 8.7% in the marine phase. Mortality in the four stocking periods varied between 7.3% and 10%. Total mortality for the two companies was 7.3% and 11.2%. Daily mortality ranged from 0% to 0.17%, with a median of 0.017% (Figure 2).

In the 121 fish-groups in subset I (first 180 days), the mean mortality was 2.7% and the median was 1.7% with a range from 0.3% to 21% (Table 1). For the fish-groups followed until harvest (subset II, $n = 74$), the mean mortality was 8.1% (median 7.0%) and varied from 2.7% to 23.9% between groups (Figure 4). Days in production at sea varied between the fish-groups from 352 to 517 days with a mean of 433 days from transfer to sea to harvest.

3.2 | Mortality classification

The grouped mortality causes of 'smolt', 'handling' and 'diseases' had a combined CSMF of nearly 60% of the registered dead fish in the full data set. Mortality due to 'handling' was the single most important group of causes (CSMF = 29.2%), followed by infectious diseases (CSMF = 17.3%) and smolt-related mortality (CSMF = 9.8%). During the first 180 days (subset I), 'smolt' mortality was the predominant cause identifying 31.7% of the registered dead fish at this point, followed by 'diseases' (17%) and 'handling' (1,5%). The differentiation in time was further evident in Figure 2, which displays the causes of death day by day in production (full data set). The 'smolt' mortality dominated the period immediately after transfer, followed by a short period of mortality due to 'diseases'. 'Handling' was the most frequent cause of death from mid production and towards the end. At the very end, the 'disease' category was again a dominant cause. In Figure 3, the mortality causes of the 74 fish-groups in subset II (harvest mortality) were sorted by cumulative mortality and the bars further divided and stacked by the different mortality causes. Here, 'smolt' mortality and 'diseases' appear to dominate the cause of death in specific groups, whereas mortality due to 'handling' is more evenly spread between the fish-groups.

3.3 | Statistical analyses

3.3.1 | Variables

Descriptive statistics of the variables for each outcome are found in Table 1. Fish-groups from self-owned hatcheries showed a lower median and variation of mortality compared with fish-groups deriving from external hatcheries (Figure 5), when relations between the outcome and the exposures in the 'early mortality' model (subset I) were investigated. The relations between temperature, days in hatchery and stocking period displayed in Figure 6 indicate an increased mortality in fish-groups when sea temperature at transfer was below 10°C, which coincided with fish stocked in spring and exceeding 350 days in the hatchery.

Treatments against salmon lice were only performed by non-medical methods. For the 'harvest mortality' (subset II), there were

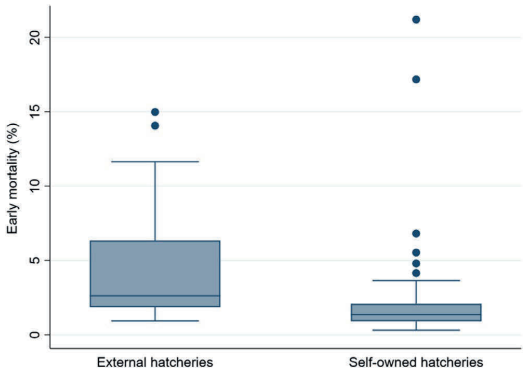

FIGURE 5 Boxplot to display the relation between early mortality (subset I, $n = 121$ fish-groups) and if the fish-group derived from a hatchery owned by the same company running the marine farm ($n = 90$ fish-groups), or if the fish-groups were bought from an external hatchery ($n = 31$ fish-groups). The line inside the box represents the median value, the box marks the values within the 25th to the 75th percentile of observations, and the upper and lower whiskers represents the respective adjacent values. Outliers are visualized as solid dots

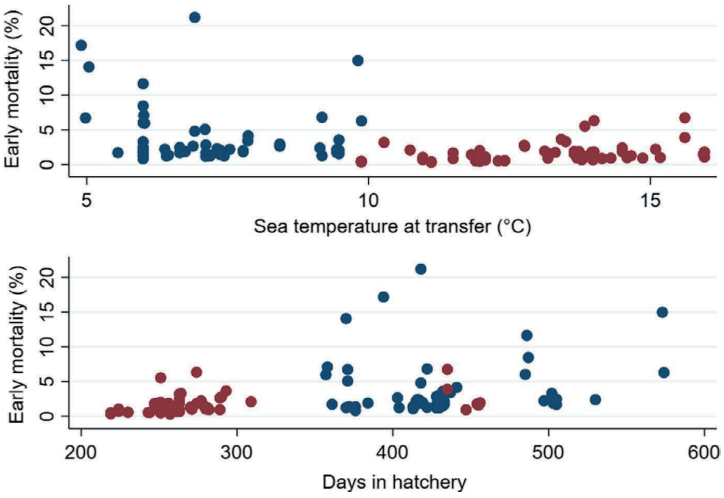

FIGURE 6 These scatterplots describe the relation between early mortality (%) and the variables (a) 'sea temperature at transfer' and (b) 'days in hatchery', in subset I (n = 121 fish-groups). In both graphs, the fish-groups are coloured blue if they were stocked in the spring and red if they were stocked in autumn

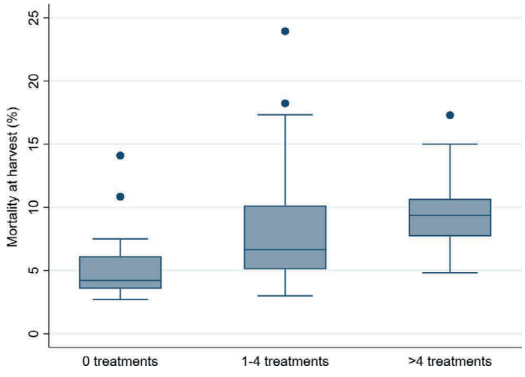

FIGURE 7 Boxplot to describe the cumulated mortality (%) at harvest (subset II, 'harvest mortality', n = 74 fish-groups) by the number of treatments against lice. The fish-groups were grouped into three treatment categories: treated 0 (n = 14 fish-groups), 1–4 (n = 42 fish-groups) or >4 (n = 18 fish-groups) times during the production. Boxplot constructed as explained in Figure 5

14 fish-groups with no treatments registered, 42 groups with one to four treatments and 18 groups with more than four treatments. In the 'early mortality', seven fish-groups had registered treatments (Table 1). Mortality was affected by the treatments, where fish-groups treated had higher mean mortality. Figure 7 shows fish-groups with different number of treatments plotted against mortality at harvest.

3.3.2 | Cross-classified multilevel modelling

Results from the final cross-classified multilevel models (CCMM) are shown in Tables 2 and 3. The fixed effects included in the final model of the ln-transformed early mortality outcome (subset I) were 'stocking period', 'temperature in sea at transfer' and 'self-owned hatchery'. For the ln-transformed harvest mortality outcome (subset II), the final model included 'delta temperature' and the categories of the number of treatments (Table 2). All fixed effects included in the final models were significant ($p < .05$).

The chain length of 100,000 iterations was sufficient for both fixed and random effects of both final models, as assessed by the Raftery-Lewis and Brooks-Draper diagnostics. Autocorrelation for fixed effects was minimal, judged by ACF and PACF. For the random effects of farm and hatchery, the PACF plots were not reduced to zero after lag 1, indicating some degree of autocorrelation for these distributions. Residual plots showed no major shortcomings in general, apart for some outliers at the farm and hatchery level deviating from the linear relationship.

3.3.3 | Sources of variation and model explanation

For the outcome variable of 'early mortality', the VCR between the intercept and the final models indicated a model explanation of 46% (Table 3). In the final model, the hatchery and farm level accounted for 23% and 6% of the VCP respectively. The variance at the fish-group level remained almost unaffected by the fixed effect (VCR: 3%) and accounted for 70% of the VCP (Table 3).

A similar pattern was observed for the fish-group level in the 'harvest mortality' model; the VCP in the final model was 70%, and VCR indicated a low model explanation (VCR: 13%). However, the remaining VCP was split the opposite way between hatchery and farm level (10% and 20% respectively). Assessed by the VCR, the fixed effects accounted for 35% of the model explanation for this outcome (Table 3).

TABLE 2 Results from the final models for the ln-transformed outcomes of 'early mortality' ($n = 121$) and 'harvest mortality' ($n = 74$). Fixed effects are displayed with β-values and SD. The categorized variable 'number of treatments' has the category of '0 treatments' as baseline

Model	'Early mortality'		'Harvest mortality'	
Number of fish-groups	121		74	
Number of farms	20		16	
Number of hatcheries	10		9	
Fixed effects	β	SD	β	SD
Stocking period	0.33	0.28		
Temperature in sea at transfer	0.1	0.04		
Self-owned hatchery	−0.77	0.26		
Delta temperature			−0.06	0.03
Number of treatments, 1–4			0.32	0.14
Number of treatments, >4			0.58	0.17
Intercept	−0.49	−0.56	1.73	0.15

TABLE 3 Variance estimates of the sources of variation in the random part of the cross-classified multilevel models for the outcomes 'early mortality' ($n = 121$) and 'harvest mortality' ($n = 74$). Results from both the random intercept models and the final models, including the variance component proportion (VCP) within each model and variance component reduction (VCR) between the intercept and final models

		Random intercept model		Final model		Model explanation
Outcome	**Sources of variation**	**Variance**	**VCP (%)**	**Variance**	**VCP (%)**	**VCR (%)**
Early mortality	Hatchery	0.33	38	0.11	23.4	67
	Farm	0.19	22	0.03	6.4	84
	Fish-group	0.35	40	0.33	70.2	6
	Total	0.87	100	0.47	100	46
Harvest mortality	Hatchery	0.05	16	0.02	10	60
	Farm	0.1	32	0.04	20	60
	Fish-group	0.16	52	0.14	70	13
	Total	0.31	100	0.2	100	35

4 | DISCUSSION

The study population had low mortality compared with national statistics (Sommerset et al., 2021). The origin of the fish affected mortality in the marine phase, and this was especially evident during the first 180 days in sea. The overall pattern seen was that smolt-related mortality dominated in the start of the marine phase, whereas mortalities related to handling or treatment were the main causes of death in total. The findings also indicate that fish-group was the most substantial source of variation in mortality, both in the early phase and when investigating the entire marine phase of production.

The grouped mortality causes ('smolt', 'handling' and 'infections') were assembled to target well-known health challenges in Norwegian salmon farming (Sommerset et al., 2021). The original causes (listed in Appendix 1) were retrieved from the farmers' production management system, and the precision is thus unknown. However, episodes of increased daily mortality are often driven by events in the production (Aunsmo et al., 2008; Aunsmo et al., 2020; Nilsen et al., 2020), for example, handling of fish (e.g. lice treatment), environmental impact (e.g. strong current) or diseases. It is reasonable to assume that mortality classification related to such known events has a high level of precision. Some diseases cannot be distinguished macroscopically (e.g. infectious pancreas necrosis and pancreas disease), and hence, it is difficult for the farmer to identify the cause of death. However, in Norway, fish health personnel must investigate cases of increased daily mortality in addition to mandatory monthly visits (Akvakultudrif tsforskriften, 2008). The findings described by fish health personnel, including disease diagnoses in the population, will help guide the site staff and thus is assumed to increase the precision of daily cause-specific mortality registrations at the farm.

In human health management, cause-specific mortality registrations are used to identify health challenges and causes of mortality in the human population (Naghavi et al., 2015, 2017; WHO, 2021). Through a standardized system, including a list of mortality causes, each death is given one cause based on the underlying cause of death (WHO, 1979, 2018). Since the system is standardized, and the list of causes is hierarchical, the results can be summarized to any population (nationally and globally) and the mortality causes can further be grouped to a relevant

level of details (WHO, 2021). Farming of salmon is also in need of information about health challenges and mortality causes in the population in order to manage the production. However, there is no standard list of mortality causes, neither a common understanding of how the information should be processed and presented. This study has, in addition to the findings, explored some methods in how data from cause-specific mortality registrations can be presented and assist in identifying challenges within health management of salmon farming.

A summary of the total number of dead fish in each cause-specific mortality group will give the farmer an overview of the variation of causes of death throughout the production. In this study, the group of smolt-related causes was high in the start of the production. In addition, out of the 10 highest daily mortality registrations in the production period, eight were within the first 20 days after the fish had been transferred to sea. Mortality identified as smolt-related was the main cause of death during six of those days. The finding is in line with the study by Bang Jensen, Qviller, et al. (2020) where salmon had the highest risk of dying during the first period in sea (Bang Jensen, Qviller, et al., 2020). When all mortality was summarized, mortality caused by handling and treatment was the predominant cause of death. However, when mortality in each fish-group was assessed at harvest, certain fish-groups had smolt-related mortality or diseases as the most important overall cause of death. This information gives valuable insight and helps identify the farms' overall challenges when it comes to mortality and where to prioritize the resources to reduce mortality.

Median annual mortality of fish-groups harvested between 2016 and 2020 in Norway was 15%–18% (Sommerset et al., 2021). The median mortality was 7% in our study. However, the variation in mortality was 3%–24% among the fish-groups. This demonstrates both the better results and further potential of the two companies were followed in this study. The variation was also evident within 'early mortality' (subset I), where the 15 fish-groups (out of 121) with the highest cumulative mortality (mean 9.7%) constituted 46% of all dead fish at this point (data not shown). This is consistent with other studies that also reported high mortality in few groups, which increased the overall mean and produced a skewed mortality pattern (Aunsmo et al., 2008; Nilsen et al., 2020). This further emphasizes the importance of considering each fish-group within each farm when investigating causes of mortality.

A CCMM was built to further investigate events affecting mortality. Using CCMM as the structure of the regression model makes it possible to build in the production hierarchy into the model, where farms can receive smolt from several hatcheries. However, the number of observations in each level of the hierarchy decreases rapidly with this approach, with potential negative effects to the robustness of the model. This was probably the cause of the increased autocorrelation and partly nonlinear relationship between residuals and normal scores at the higher levels of the models, which results in some uncertainty regarding the estimates of the random effects in this study.

The choice of analysing 'early mortality' and 'harvest mortality' as two separate models was made to identify any differences in how the explanatory variables might affect the mortality differently during the marine production. This contributes to the validity of the model setup, since the importance of the fish origin before sea transfer decreases gradually during the time in production. Looking at the random effects of 'farm' and 'hatchery' in the intercept models (no fixed effects included), the model with 'early mortality' as outcome placed most variance at the 'hatchery' level, whereas 'harvest mortality' outcome had most variance at the 'farm' level. This supports our theory that time in production (together with the production hierarchy) is important when building such models. In the final models, where the fixed effects are accounted for, the variance in the random part was reduced at both 'hatchery' and 'farm' level. However, the variance at the 'fish-group' level remained almost unchanged but with an increase in VCP to 70%. This indicates that the fixed effects of the models explained events occurring at the 'hatchery' and 'farm' level, but to a lesser degree have an impact to each individual fish-group, meaning the variables available had limited ability to identify the causes of mortality at the 'fish-group' level. Starting point for this study was one of the most detailed set of variables possible to obtain from the production system used in the salmon farming today. Hence, the study shows a need for more detailed knowledge to each fish-group in order to explain the 70% of variation in the model detected at this level with this approach.

Among the fixed effects, stocking period was the variable with most effect on mortality in the 'early mortality' model. Fish stocked in spring had increased mortality compared with fall-stocked fish. This is similar to findings from earlier studies (Bang Jensen, Qviller, et al., 2020; Nilsen et al., 2020; Pincinato et al., 2021). Sea temperature at transfer was also significant in the model, indicating an increase in mortality when the temperature rises. However, the temperature at transfer alone in the model was not significant, and graphical assessment of the relationship showed that mortality decreased with fish stocked during spring, along with rising temperatures, while fish stocked in autumn had the highest mortality at the highest temperatures. These variables are also related to 'days in hatchery' (Figure 6), where fish-groups transferred to sea at temperatures below 10°C and after more than 350 days at the hatcheries are stocked in spring. In addition, temperature will be an indicator of when in the stocking period the fish was transferred to sea, and low temperatures indicate stocking either early in the spring or late in autumn for example. Temperature, stocking period, season of year and days in hatchery all affect the mortality; however, causality is difficult to establish.

Four of the ten hatcheries in this study were owned by the companies who ran the marine farms. Fish-groups deriving from these four hatcheries ($n = 90$) had a lower mean mortality at 180 days than the remaining 25% ($n = 31$) of the fish-groups deriving from the other six hatcheries. The variable ('self-owned hatcheries') was included in the final model for early mortality outcome. This indicates a lower early mortality if the farming company owns both the hatchery and the marine farms. At large, this could be interpreted as if you are in control of the entire life of the fish; you are in a better position to control the production at the hatchery and reduce mortality in the early stage of the production in sea.

As described by others (Bang Jensen, Qviller, et al., 2020; Salama et al., 2016; Soares et al., 2011), this study also emphasizes the high risk of mortality in the first period after transferring the smolt to sea. A study by Pincinato et al. (2021) identified smolt-related mortality (or smolt quality) as important when analysing factors affecting losses in Norwegian aquaculture through a questionnaire-based survey performed in 2011, based on farm-level data (Bleie & Skrudland, 2014; Pincinato et al., 2021). They further associated these production losses to differences between hatcheries. The sources of variation in our study, based on cage-level data, identified the fish-group to be more important than the hatchery, where fish-groups with an increased early mortality in sea were spread between the hatcheries. This is important, since it requires a more detailed monitoring and recording of the fish health status of each fish-group in order to further reveal the causes of mortality. Measures taken across fish-groups at a hatchery or farm will not necessarily be sufficient.

Looking at the final model of 'harvest mortality', the number of salmon lice treatments drives the mortality and none of the significant fixed effects in the early mortality model remained significant at this stage. Treatments have been reported from several other studies to be the main cause of mortality in the Norwegian salmon farming during the last years (Bang Jensen, Qviller, et al., 2020; Overton et al., 2019; Sommerset et al., 2021) as well as a recent study showing increased mortality especially from non-medical treatments when investigating lice treatments in detail (Sviland Walde et al., 2021). Our study supports these findings of high mortality associated with lice treatments, also by identifying 'handling' as the most prevalent group of mortality causes in terms of number of dead fish. The CCMM indicates that mortality in the fish-group increases with the number treatments. However, future studies should also include details of which type of non-medical treatment method (preferably down to which vessel) used to further increase knowledge about mortality related to treatment. This information could also be integrated as a part of the mortality classification system, identifying not only 'lice treatment' as a cause of death, but the actual method or vessel used in the treatment. The farmer would then have access to detailed information, explaining causes of mortality related to lice treatments, as an integrated part of the health management.

Apart from the treatment variables, the 'delta temperature' was also significant in the final model of harvest mortality, meaning the difference in water temperature from the hatchery to the sea at transfer affected the mortality at the end of production. The measured effect on mortality was limited according to the model, but the effect was robust and stable throughout the modelling work. However, the causal pathway of the effect of delta temperature on harvest mortality remained unclear, and this result should be interpreted cautiously.

The study population consisted of fish-groups of salmon within two companies, making the number of study units relatively few when the salmon farming industry in total is considered the target population. Hence, the results should be interpreted with care and with considerations of the limitation in external validity. Internal validity (validity of the causal relationships presented from the models) is considered adequate. The study is based on the data possible to retrieve retrospectively at this resolution today. However, the validity would have been improved further if more measurements (and traceability of the study unit) in the production of salmon were standardized in time and space. As of today, the authors regard this as a limitation in epidemiological studies comparing fish-groups across salmon farming companies. The structure of data in Norwegian fish farming favours 'farm' as the epidemiological unit (Bang Jensen, Mårtensson, et al., 2020; Bang Jensen, Qviller, et al., 2020); however, this study shows that cage to cage variation is important. Hence, it is difficult to produce studies of fish-groups with an increased validation and applicable results beyond the study population. Results from this study emphasize the importance (statistical and biological) of fish-groups within the farm, meaning improvement of structure and traceability of data are necessary to further investigate biological variation between fish-groups, not only between farms.

This study has shown that daily cause-specific mortality records can be used to effectively describe mortality patterns at a chosen unit and time in salmon farming. This have the potential to be an important tool within fish health management. Furthermore, sources of variation deriving from cross-classified multilevel modelling can be used when analysing causes of mortality and identify which part of the production hierarchy contributes the most. The fish-group attributed 70% of the variation in mortality in this study. This points towards the need for more detailed information from each fish-group to further investigate the causes of mortality in the sea phase. Overall in the production, the mortality due to handling of fish and treatment of salmon lice was the major cause of death. However, the cause-specific mortality classification registrations identified smolt-related causes to be the major cause of death during the first 180 days and for specific fish-groups also when mortality was summarized at harvest. This means that targeted preventive strategies against mortality at the fish-group level are important to increase survival, improve fish welfare and improve production of farmed salmon.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

The data set generated in the study is not included but is available from the corresponding author on reasonable request.

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REFERENCES

Akvakultudriftsforskriften. (2008). Forskrift om drift av akvakulturanlegg. https://lovdata.no/forskrift/2008-06-17-822/

Aunsmo, A., Bruheim, T., Sandberg, M., Skjerve, E., Romstad, S., & Larssen, R. B. (2008). Methods for investigating patterns of mortality and quantifying cause-specific mortality in sea-farmed Atlantic salmon *Salmo salar*. *Diseases of Aquatic Organisms*, *81*(2), 99–107. https://doi.org/10.3354/dao01954

Aunsmo, A., Øvretveit, S., Breck, O., Valle, P. S., Larssen, R. B., & Sandberg, M. (2009). Modelling sources of variation and risk factors for spinal deformity in farmed Atlantic salmon using hierarchical- and cross-classified multilevel models. *Preventive Veterinary Medicine*, *90*(1), 137–145. https://doi.org/10.1016/j.prevetmed.2009.03.015

Aunsmo, A., Stormoen, M., Persson, D., & Midtlyng, P. J. (2020). *Klassifisering av taps og dødsårsaker i akvakultur – forprosjekt*. NMBU. ISBN: 978-82-575-1711-3.

Bang Jensen, B., Mårtensson, A., & Kristoffersen, A. B. (2020). Estimating risk factors for the daily risk of developing clinical cardiomyopathy syndrome (CMS) on a fishgroup level. *Preventive Veterinary Medicine*, *175*, 104852. https://doi.org/10.1016/j.prevetmed.2019.104852

Bang Jensen, B., Qviller, L., & Toft, N. (2020). Spatio-temporal variations in mortality during the seawater production phase of Atlantic salmon (*Salmo salar*) in Norway. *Journal of Fish Diseases*, *43*(4), 445–457. https://doi.org/10.1111/jfd.13142

Bleie, H., & Skrudland, A. (2014). *Tap av Laksefisk i sjø*. Mattilsynet. https://www.mattilsynet.no/fisk_og_akvakultur/fiskevelferd/tap_av_laksefisk_i_sjo_2014.15430

Browne, W. J. (2019). *MCMC estimation in MLwiN v3.03*. MLwiN manuals. http://www.bristol.ac.uk/cmm/software/mlwin/download/manuals.html

Charlton, C., Rasbash, J., Browne, W. J., Healy, M., & Cameron, B. (2020). *MLwiN version 3.05 Centre for Multilevel Modeling*. University of Bristol.

Dohoo, I. R., Tillard, E., Stryhn, H., & Faye, B. (2001). The use of multilevel models to evaluate sources of variation in reproductive performance in dairy cattle in Reunion Island. *Preventive Veterinary Medicine*, *50*(1), 127–144. https://doi.org/10.1016/S0167-5877(01)00191-X

Gåsnes, S. K., Oliveira, V. H. S., Gismervik, K., Ahimbisibwe, A., Tørud, B., & Jensen, B. B. (2021). Mortality patterns during the freshwater production phase of salmonids in Norway. *Journal of Fish Diseases*, *44*(12), 2083–2096. https://doi.org/10.1111/jfd.13522

Houe, H., Gardner, I. A., & Nielsen, L. R. (2011). Use of information on disease diagnoses from databases for animal health economic, welfare and food safety purposes: Strengths and limitations of recordings. *Acta Veterinaria Scandinavica*, *53*(1), S7. https://doi.org/10.1186/1751-0147-53-S1-S7

Iversen, M., Finstad, B., McKinley, R. S., Eliassen, R. A., Carlsen, K. T., & Evjen, T. (2005). Stress responses in Atlantic salmon (*Salmo salar* L.) smolts during commercial well boat transports, and effects on survival after transfer to sea. *Aquaculture*, *243*(1), 373–382. https://doi.org/10.1016/j.aquaculture.2004.10.019

Johansson, L.-H., Timmerhaus, G., Afanasyev, S., Jørgensen, S. M., & Krasnov, A. (2016). Smoltification and seawater transfer of Atlantic salmon (*Salmo salar* L.) is associated with systemic repression of the immune transcriptome. *Fish & Shellfish Immunology*, *58*, 33–41. https://doi.org/10.1016/j.fsi.2016.09.026

Leckie, G., & Charlton, C. (2012). runmlwin: A program to run the MLwiN multilevel modeling software from within Stata. *Journal of Statistical Software*, *52*(11). https://doi.org/10.18637/jss.v052.i11

McCormick, S. D. (2012). Smolt physiology and endocrinology. In S. D. McCormick, A. P. Farrell, & C. J. Brauner (Eds.), *Euryhaline fishes* (Vol. *32*, pp. 199–251). Academic Press.

Naghavi, M., Abajobir, A. A., Abbafati, C., Abbas, K. M., Abd-Allah, F., Abera, S. F., Aboyans, V., Adetokunboh, O., Afshin, A., Agrawal, A., Ahmadi, A., Ahmed, M. B., Aichour, A. N., Aichour, M. T. E., Aichour, I., Aiyar, S., Alahdab, F., Al-Aly, Z., Alam, K., … Murray, C. J. L. (2017). Global, regional, and national age-sex specific mortality for 264 causes of death, 1980–2016: A systematic analysis for the Global Burden of Disease Study 2016. *The Lancet*, *390*(10100), 1151–1210. https://doi.org/10.1016/S0140-6736(17)32152-9

Naghavi, M., Wang, H., Lozano, R., Davis, A., Liang, X., Zhou, M., & Vollset, S. E. (2015). Global, regional, and national age–sex specific all-cause and cause-specific mortality for 240 causes of death, 1990–2013: A systematic analysis for the Global Burden of Disease Study 2013. *The Lancet*, *385*(9963), 117–171. https://doi.org/10.1016/S0140-6736(14)61682-2

Nilsen, A., Nielsen, K. V., & Bergheim, A. (2020). A closer look at closed cages: Growth and mortality rates during production of post-smolt Atlantic salmon in marine closed confinement systems. *Aquacultural Engineering*, *91*, 102124. https://doi.org/10.1016/j.aquaeng.2020.102124

Overton, K., Dempster, T., Oppedal, F., Kristiansen, T. S., Gismervik, K., & Stien, L. H. (2019). Salmon lice treatments and salmon mortality in Norwegian aquaculture: A review. *Reviews in Aquaculture*, *11*(4), 1398–1417. https://doi.org/10.1111/raq.12299

Pincinato, R. B. M., Asche, F., Bleie, H., Skrudland, A., & Stormoen, M. (2021). Factors influencing production loss in salmonid farming. *Aquaculture*, *532*, 736034. https://doi.org/10.1016/j.aquaculture.2020.736034

Salama, N. K. G., Murray, A. G., Christie, A. J., & Wallace, I. S. (2016). Using fish mortality data to assess reporting thresholds as a tool for detection of potential disease concerns in the Scottish farmed salmon industry. *Aquaculture*, *450*, 283–288. https://doi.org/10.1016/j.aquaculture.2015.07.023

Soares, S., Green, D. M., Turnbull, J. F., Crumlish, M., & Murray, A. G. (2011). A baseline method for benchmarking mortality losses in Atlantic salmon (*Salmo salar*) production. *Aquaculture*, *314*(1), 7–12. https://doi.org/10.1016/j.aquaculture.2011.01.029

Sommerset, I., Bang Jensen, B., Bornø, B., Haukaas, A., & Brun, E. (2021). *Fish health report 2020*. Norwegian Veterinary Institute. https://www.vetinst.no/en/reports-and-publications/reports

Sørensen, H. T., Sabroe, S., & Olsen, J. (1996). A Framework for evaluation of secondary data sources for epidemiological research. *International Journal of Epidemiology*, *25*(2), 435–442. https://doi.org/10.1093/ije/25.2.435

Sviland Walde, C., Bang Jensen, B., Pettersen, J. M., & Stormoen, M. (2021). Estimating cage-level mortality distributions following different delousing treatments of Atlantic salmon (*Salmo salar*) in Norway. *Journal of Fish Diseases*, *44*(7). https://doi.org/10.1111/jfd.13348

Tørud, B., Bang Jensen, B., Gåsnes, S., Grønbech, S., & Gismervik, K. (2019). *Dyrevelferd i settefiskproduksjonen [Animal welfare in the hatcheries]*. Norwegian Veterinary Institute. https://www.vetinst.no/rapporter-og-publikasjoner/rapporter/2019/dyrevelferd-i-settefiskproduksjonen-smafiskvel (in Norwegian)

World Health Organization, WHO. (1979). *Medical certification of cause of death, instructions for physicians on use of international form of medical certificate of cause of death*. World Health Organization. https://apps.who.int/iris/handle/10665/40557

World Health Organization, WHO. (2018). *International classification of diseases for mortality and morbidity statistics* (11th Revision ed.). https://icd.who.int/en

World Health Organization, WHO. (2021). *World health statistics 2021: Monitoring health for the SDGs, sustainable development goals*. World Health Organization. https://apps.who.int/iris/handle/10665/342703

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APPENDIX 1

Overview of all mortality causes included in the constructed groups from the 'full data set'. Displayed as both the actual number of dead fish for each cause, the cause-specific mortality fraction (CSMF) of the total number of dead fish (%) and the corresponding grouped CSMF used in this study. CSMF of less than 0.1% was for readability set to '<0.1%'. The mortality causes are sorted descending by the prevalence within each group. In the raw data (in Norwegian), some mortality causes occurred multiple times, only with differences in spelling or different acronyms used towards the same cause. When translated into English, those causes were merged. For traceability, merged causes have the number of original variables (2 or 3) in brackets behind the name. This also emphasize the need of a common standard of mortality causes in salmon farming.

Grouped mortality cause	Mortality cause	Number of dead fish	CSMF of total number of dead fish (%)	Grouped CSMF of total number of dead fish (%)
Smolt-related	Incomplete smoltification (2)	82,605	4.6 %	9.80%
	Transportation	46,625	2.6 %	
	Dead at arrival	25,323	1.4 %	
	Nephrocalcinosis	13,147	0.7 %	
	Fin damage	7,044	0.4 %	
	Haemorrhagic smolt syndrome (HSS) (2)	778	<0.1%	
Infectious diseases	Tenacibaculum	81,958	4.6 %	17.30%
	Cardiomyopathy syndrome (CMS) (2)	74,031	4.1 %	
	Heart and skeletal muscle inflammation (HSMI) (2)	73,636	4.1 %	
	Pancreas disease (PD) (2)	70,150	3.9 %	
	Infectious pancreatic necrosis (IPN) (2)	6,737	0.4 %	
	Yersinia infection (2)	3,512	0.2 %	
	Mouth rot	1,399	<0.1%	
	Fungal infection	413	<0.1%	
Handling and lice treatment	Handling (2)	243,264	13.5 %	29.20%
	Lice treatment (2)	238,728	13.3 %	
	Grading	34,317	1.9 %	
	Moving grading	7,714	0.4 %	
	Bath treatment	1	<0.1%	

Grouped mortality cause	Mortality cause	Number of dead fish	CSMF of total number of dead fish (%)	Grouped CSMF of total number of dead fish (%)
Other	Unknown (2)	376,206	20.9 %	43.70%
	Undefined	111,192	6.2 %	
	Runts (3)	81,664	4.5 %	
	Ulcer (3)	71,295	4.0 %	
	Sexual maturation (2)	31,948	1.8 %	
	Winter ulcer	30,388	1.7 %	
	Normal	23,261	1.3 %	
	Birds—cormorant	9,685	0.5 %	
	Proliferative gill infection (PGI)	8,093	0.5 %	
	Injuries (2)	7,612	0.4 %	
	Fin rot	7,522	0.4 %	
	Old	4,871	0.3 %	
	Amoebic gill disease (AGD)	4,870	0.3 %	
	Deformities (2)	4,531	0.3 %	
	Other gill problems	2,324	0.1 %	
	Dead due to incidents	2,084	0.1 %	
	Culled (2)	1,926	0.1 %	
	Gill infection	1,676	<0.1%	
	Predators (3)	1,396	<0.1%	
	Discarded	1,217	<0.1%	
	Suspected disease	960	<0.1%	
	Sampling	928	<0.1%	
	Egg not fertilized	284	<0.1%	
	Gill infection—other	89	<0.1%	
	Technical failure	52	<0.1%	
	Birds—heron	11	<0.1%	
Total	Total number of dead fish	1,797,467	100%	100%

Paper II

Real-time monitoring of cause-specific mortality- and losses in industrial salmon farming

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Abstract

Mortality represents a major challenge in farming of salmon in open sea cages. Production takes place in large cages with up to 200 000 fish and offers no other treatment of individual fish than euthanasia of moribund fish. Whole cages can be treated with therapeutics or mechanically for salmon lice, but history have demonstrated treatment as non-effective strategies in reducing overall mortality.

Current trends in research on mortality and losses are increasingly focusing on describing pathology and potentially reduced fish welfare, without emphasis on innovative means for effectively reducing the problem.

The described project was initiated with the objective to develop a standardized system for classification of mortality and losses in aquaculture. The WHO`s International Classification of Disease (ICD) has served as a model tool, and where adaptation into an animal production system will be useful.

The current paper describes our proposal for a standard system for classification and monitoring of mortality and losses in industrial aquaculture, covering the full production cycle from eggs to harvest. The system is based on causality with the underlying cause of death as the principal variable to monitor. The system describes the fish-farmer as the primary user of a system, but with additional opportunities for the supply industry, authorities, and research to utilize anonymized data. The classification code is organized in a hierarchical structure with three levels: six main categories, causal subcategories at level 2, and in principle, unlimited underlying causes at level three. The hierarchy allow both registration and retraction of data at the three different levels, including registration of unspecified causal categories at level one and two.

The proposed code is conceptuated to also allow classification of other losses than mortality, thus being able to monitor the effect of the underlying cause for all areas of losses including quality downgrading, reduced growth, reduced feed conversions etc. A code covering all areas of losses will enable the fish farmer to monitor the total effect underlying causes, both in biological and monetary terms. In this way it will be possible to target limited resources in the direction of losses with the highest impact and the interventions with the highest pay-back. By establishing a unique code, it will be possible to standardize registrations between area of losses within a company, between companies and regions, and potentially also between countries.

Key words: Salmon farming, cause-specific mortality, causality, classification, monitoring

1. Introduction

Salmon farming is a relatively new aquaculture production which started in Norway in the late 1960`s. The industry has developed rapidly and today there is a substantial production in countries with cold seawater regions of Europe, North- and South America (Iversen, Asche, Hermansen, & Nystøyl, 2020). Health related losses and especially high mortality have been and still are major obstacles, challenging the further development of a sustainable industry (Persson, Nødtvedt, Aunsmo, & Stormoen, 2021; Sommerset, Bang Jensen, Bornø, Haukaas, & Brun, 2021)

Norwegian salmon farming has experienced an increase in production cost from NOK 25 per kg gutted salmon in 2008 to NOK 47,80 in 2020 (Directorate of Fisheries, 2021). There has been some increase in expenditures, and especially feed prices, but a large share of this increase is due to biological inefficiency in production (Iversen et al., 2020). This picture is similar in most salmon producing countries. To reduce this production inefficiency and capitalize more of the potential in salmon farming targeted interventions on the underlying causes of losses are essential.

Ellis et al. (2012) stated that fish do not just die – there has to be a cause. Using methods in fish health, epidemiology and other sciences it is possible to describe and analyze mortality and losses in aquaculture, both quantitively and qualitatively (Aunsmo et al., 2008; Aunsmo, Valle, Sandberg, Midtlyng, & Bruheim, 2010; Bang Jensen, Qviller, & Toft, 2020; Persson et al., 2021; Pettersen, Rich, Jensen, & Aunsmo, 2015). However, it requires an understanding of the farming system and involvement of stakeholders to turn this knowledge into practical strategies and improvement (Turnbull et al., 2011). To achieve improvement it is therefore essential to communicate and combine scientific knowledge and operational skills into new and improved ways of management. Establishing s and monitoring the causes of mortality and losses is therefore critical for targeted and cost-effective use of resources in salmon farming.

In human medicine, reliable and timely information on cause-specific mortality is described as fundamental for informing the development, implementation, and evaluation of health policy as well as how to allocate resources in the nation's health care system (Naghavi et al., 2017; World Health Organization, 2021b). The International Classification of Disease (ICD) has developed over centuries to serve as an important tool in health management and has become an important part of the World Health Organization (World Health Organization, 2019, 2021a).

In terrestrial farmed animals there are some specific studies on cause- specific mortality of farmed pigs and cattle to be found (MK Shoo 1992, JP Vaillancourt 1990, Christensen 1995, Koketsu 2006). However, these are situation specific studies and there is general a lack of information about systematic monitoring of cause- specific mortality in terrestrial animal farming. This contradiction is

somewhat surprising regarding its pivotal importance in human medicine and health management. This lack of information on both real- time and historical mortality causes will reduce precision of health policy of farmed animals at both the national level and at the industry, farm, or company level.

Total daily mortality at cage-level is monitored in most salmon producing countries and used in farm management. In several countries' (Norway, Chile, Scotland, Faroe Island, Iceland and Scotland) mortality records are also reported to the authorities in line with regulations (Akvakulturdriftsforskriften, 2008; Faroese Food and Veterinary Authority, 2019; Icelandic Food and Veterinary Authority, 2020; Marine Scotland, 2009; Sernapesca, 1995). A few studies on cause-specific mortality and reports on the main causes of death of farmed salmon exist (Aunsmo et al., 2008; Persson et al., 2021; Pincinato, Asche, Bleie, Skrudland, & Stormoen, 2021), while statutory reporting is generally limited to total mortality figures. In industrial salmon farming the companies and units are large, favoring systematic health management based on detailed data on cause-specific- losses and mortality along the value chain. Many salmon companies do register cause-specific mortality; however, the classification is based on a mix of causes, clinical signs and risk factors, and there is no standardized code system.

There is limited or no treatment of individual animals in salmon farming, which differs from human medicine, pet animals and some terrestrial farmed animal species. This principal difference has implications for how monitoring should be structured as it requires more focus on populations to make changes and improvements in production and fish welfare.

The aim of this work was to develop a standard for classification of cause-specific mortality and losses in industrialized aquaculture, based on causality as the bearing principle. The ambition has been to make the classification system comprehensive enough to – with time – encompass all aspects of biological losses within the production, from egg to harvest.

2. Review of current methods for classification of mortality and morbidity

The project has used mortality and morbidity classification systems in human medicine, terrestrial farming in Scandinavian countries, and current practices in salmon farming as background material for the work.

2.1. Cause specific mortality in human health management

The classification of mortality and morbidity in humans has a history of several hundred years (Knibbs, 1929). The first International List of Causes of Death was adopted by the International Statistical Institute in 1893, and the recent ICD-11 was adopted by the WHO May 2019 and will come

into effect January 2022 (World Health Organization, 2019, 2021a). The ICD standard is based on causality where the "Underlying Cause of Death" is the basis of mortality classification (Brooke et al., 2017; World Health Organization, 1979). ICD contains more than 17 000 diagnostic categories and 100 000 medical terms. The classification is based on a hierarchical system with 28 main categories (Chapters), and where the single underlying cause may only belong to one chapter. Further subdivisions are found where for instance chapter 1 Infectious diseases are sub-divided into the type of infectious agent such as bacteria, viruses, helminths, arthropods etc. External causes (chapter 23) encompass causes defined as environmental events and circumstances that cause injuries and other adverse effects (World Health Organization, 2019). The purpose of the ICD is defined as "To allow the systematic recording, analysis, interpretation and comparison of mortality and morbidity data collected in different countries or areas and at different times" (Naghavi et al., 2017; World Health Organization, 2021b).

For the principles and structure of our proposed system for salmonid aquaculture, the ICD in human medicine provided essential and dominant scientific references, scrutinizing publications discussing advantages and challenges on essential principal and technical questions. In particular with respect to the definition and use of the underlying cause as the principal classification criteria (Brooke et al., 2017; Brooks & Reed, 2015; World Health Organization, 1979, 2019). Also structuring of the system to allow aggregation of records, spatially and temporally, not only according to single causes but also to form relevant groupings up to main categories (infectious, traumata, environmental influences etc.) (Knibbs, 1929; Naghavi et al., 2017). The total ICD system also describes procedures for, and experiences of, using information from persons outside the medical and diagnostic professions in assigning mortality causes (Murray et al., 2014; Serina et al., 2016; Soofi et al., 2015; World Health Organization, 2016).

Obviously, there are some important differences between ICD and a system designed for use in animal production, e.g., focus of individuals in human medicine vs. on populations in farmed animals; the use of euthanasia in animal husbandry, and the option to include causes of inferior commercial quality (downgrading) of harvested carcasses, and morbidity effects (growth and feed conversion) into the system for aquaculture, age of individuals, and not at least available resources for health care.

Verbal autopsy (VA) is a method to ascertain cause of human deaths based on information on symptoms, signs and circumstances obtained from the deceased`s caretakers (Soleman, Chandramohan, & Shibuya, 2006; World Health Organization, 2016). Verbal autopsy has been developed as a tool to secure vital information of health especially in developing countries, where

death often occurs at home and with limited contact with medical personnel or hospitals and no standard medical death certificate. The VA method is based on standardized tools comprising questionnaire for interviews, mortality classification system, and diagnostic criteria for deriving to a cause of death when the answers are interpretated by either a physician or an algorithm (Murray et al., 2014; Soleman et al., 2006). The aim of using VA in human populations is to estimate the relative contribution of different mortality causes in absence of the "gold standard" method (e.g., autopsy or hospital records). We found verbal autopsy useful as a methodology when considering how to develop a system for cause-specific mortality in industrial aquaculture. Since autopsy of each dead fish is not feasible, verbal autopsy resembles the practice of establishing cause of death in aquaculture.

2.2. Mortality and disease monitoring in Norwegian aquaculture

The second important material for developing the proposed system outline came from the existing practice of generating mortality and disease records in Norwegian salmon companies. Daily records of mortality and losses have been included in current production management software for salmon production since the late 1980'ies, allowing review and analysis on site- and company level, and reported into a national database since 1994 (Directorate of Fisheries, 2022). To describe status quo, a questionnaire survey of mortality categories being currently used in nine Norwegian salmon farming companies (large, medium, and small enterprises) was carried out. The number of categories ranged from 20 to 50 (median value=28); in total giving 103 different mortality categories falling into 15 main mortality groups. The greatest diversity of mortality categories was found in the main group "miscellaneous" (n=17), "treatments" (n=16) and "environment" (n=11). While a number of mortality categories were distinct diagnoses (e.g. "Yersiniosis", "Pancreas Disease") others were "mixed bags" based on visual appearance (e.g. "wounds", "pinheads") or named by risk factors (e.g. "smolt related mortality", "mortality related to vaccination", etc.). Despite the diversity seen in the naming of mortality categories, the project group came to the conclusion that harmonization towards joint, uniform mortality categories to be used across farming companies was achievable, given necessary investment in professional time and expertise. Aunsmo et al. (2008) found that in 20 cages in 10 farms, the cause of death could be classified into 22 underlying causes for 92% of the mortalities. This shows the potential for establishing a standardized system for classifying mortality into underlying cause of death in aquaculture.

Publication of data from diagnostic and analytical laboratories by the Norwegian Veterinary Institute (NVI) were investigated; in particular the reports on fish health and welfare issued retrospectively each year (Sommerset et al., 2021). Periodic updates on the notifiable fish diseases are also provided on the NVI website, and also form the basis for online publication and identification of sites that are

suspected of certain notifiable diseases on the Barentswatch website (https://www.barentswatch.no/fiskehelse/). The outcomes of diagnostic investigation by both governmental and commercial veterinary laboratories form the basis for these data, where diagnosis upon notification of suspicion or surveillance activities for the notifiable fish diseases has the main focus. While the information on the prevalence and geographical spread of the notifiable diseases and infections has high quality, this source cannot quantify the associated losses and further lacks empirical data on the non-notifiable infections and on non-infectious causes of mortality -and losses. Recent years, questionnaire surveys among fish health personnel working in the field has been carried out to compensate for this shortage, giving a priority ranking of the most important health issues in Norwegian fish farming (Sommerset et al., 2021).

At events of increased mortality at the farm fish health professionals investigate the mortality through qualitative clinical and pathological examinations, submit samples to laboratories for histopathological- or infectious agents diagnostics. However, the investigation into the pathogenesis of disease has its limitations in revealing causality of disease. The project group identified a shortage in systematic investigations into the causality of mortality in Norwegian salmon farming.

The system for registration cause-specific mortality is currently already in use within several salmon producers. Fishtalk© (AKVA group) and Mercatus© (Scale AQ), the leading production management systems, have technical solutions for daily assigning causes of death per cage.

2.3. Monitoring mortality and causes in international salmon farming

Data systems for monitoring and reporting health issues and can either be authority regulated or being industry operated. Authority regulated systems do often have annual reports, industrial operated systems do more rarely publish, while specific scientific projects publish most studies.

Scientific papers reporting mortality monitoring from industrial salmon aquaculture operations are some, but sporadic over countries and years, and being outcomes of time-limited projects. Ireland (Crockford, Menzies, McLoughlin, Wheatley, & Goodall, 1999; Menzies, McLoughlin, Wheatley, & Goodall, 1996; Wheatley, McLoughlin, Menzies, & Goodall, 1995). Norway (Aunsmo et al., 2008; Brun, Poppe, Skrudland, & Jarp, 2003). Scotland (Kilburn et al., 2012; Soares, Green, Turnbull, Crumlish, & Murray, 2011; Soares, Murray, Crumlish, Turnbull, & Green, 2012; Soares, Murray, Crumlish, Turnbull, & Green, 2013). Canada (Karreman, 1991).

In Scotland , fish farms production surveys are published annually by Marine Scotland Science (Marine Scotland, 2021). These reports do not contain information on mortality directly, but the total losses of salmon and rainbow trout from sea transfer to harvest is provided indirectly, as a

proportion of each smolt yearclass that has been harvested since sea transfer. This data series goes back more than 40 years, since 1979. Like in Norwegian public data, there is no specification regarding the contribution of different causes to the observed non-survival.

In 2003 Faroe Islands implemented new veterinary legislation comprising both mandatory monthly reporting, and routine on-site inspections with comprehensive diagnostic sampling and -analysis by the governmental food safety laboratory (Faroese Food and Veterinary Authority, 2019; Faroese seafood, 2022). The results are summarized and published in retrospect during annual aquaculture conferences, showing the historic development in total mortality and prevalence for specified seven viral and two bacterial infections (predominantly based on RT-PCR screening results). The data do present the proportion of sites with clinical disease, but no estimation of their importance in terms of biological or economic losses.

Icelandic regulation requires fish-farmers to monthly submit mortality numbers per cage into a national database, there is no compulsory cause- specific monitoring (Icelandic Food and Veterinary Authority, 2020). Real time data on monthly mortality (%) and fish stock are available per site at the website for the Icelandic Food and Veterinary Authority MAST.

However, likely the most advanced system for regulatory reporting of fish losses can be found in Chilean salmon culture. The data are based on weekly reporting from all fish farming sites to the national fisheries authorities (Sernapesca, 1995). The number of fish lost from marine farming sites are assigned between 10 categories on category 1 (non-infectious causes), and 18 categories on category 2 (infectious diseases) (Sernapesca, 2012). These data that are being summarized and published through written reports after the first 6 months, and at the end of each year are available on-line (Sernapesca, 2021). Chile is the only salmonid farming region that provides empirical, industry-wide and publicly available monitoring data showing the relative contribution of the most important both infectious and non-infectious diseases to the overall mortality.

In Chile, the representatives from the aquaculture supply industry have contributed importantly towards standardisation of and education in mortality assignment though a published manual covering both category 1 and category 2 causes (Elanco, 2020). Also in Norway, a book volume and a poster (in Norwegian) are being distributed, covering 33 of the most common causes of mortality in Norwegian salmon aquaculture (Marinhelse, 2018).

2.4. Cause specific mortality registration in terrestrial animal farming

Norway was chosen for comparison because of the same regulatory system as our base case aquaculture production, and Denmark because of the same scale of pork production as the Norwegian salmon industry.

Norway has a common reference coding system for diseases for livestock production of cattle, sheep, goats, pigs and poultry (Animalia, 2021). This code system is linked to the Norwegian Food Safety Authority's lists of notifiable diseases in Norway (A, B and C diseases) (Forskrift om sykdom hos dyr, 2015). The coding system is made up by different interests such as the public disease list, disease agents, organ-specific disorders, species-specific disorders, etc. For infectious diseases, it is the agent that indicates disease. For the organ-specific diseases, it is the pathological damage that indicates the disorder. There are a few codes related to the environment (suffocation), some codes are related to treatment (sterilization, treatment by deworming, etc.). There are no codes specifically related to mortality. The coding system is mainly for the purpose of reporting diagnoses, diseases, and treatment.

Pet animals in Norway have a national database (https://pyramidion.no/) with a reference coding system which animal hospital voluntarily can join and report to. As for livestock the purpose is to report diagnoses, diseases and treatments of the individual animal. No specific codes related to causes of mortality are included.

In Danish pig production, mortality or the cause of mortality is not registered in a central digital system. Mortality is registered by the destruction facility DAKA (https://www.daka.dk/dk/daka/om-os/) where the number of deaths in the category's sows, slaughter pigs and piglets are registered (Tina Birk Jensen, personal communication). There is no public database of disease registrations, but the animal owner is obliged to keep an overview of medicine use with information about animals, diagnosis, medicine, and medicine consumption. This overview can be kept in paper and checked by the Danish Veterinary and Food Administration. Veterinarians are obliged to report the prescribed amounts of medicines to a central database called VetStat (with public information of drug use per herd (VetStat, 2021).

Table 1. Summary of registration of mortality, cause-specific mortality and treatments in human medicine and different animal production systems in Norway. In addition, also pork production in Denmark due to similarities in size and national economic importance as salmon in Norway.

	Mortality numbers		**Cause- specific mortality**		**Disease treatment**	
System	National database	Company-database*	National database	Company-database*	National database	Company-database*
ICD (human)	yes	yes	yes	unclear	no	yes
Salmon Norway	yes	yes	no	yes	partly	yes
Dairy Norway	yes	yes	partly	partly	yes	yes
Poultry Norway	yes	yes	partly	partly	yes	yes
Pork Denmark	yes	yes	no	no	yes	yes
Pet animals Norway	no	no	no	no	yes	yes

* Institutions like hospitals, homes for elderly, doctor offices for human medicine

2.5. Working methods in the project

The full project was established with a pre-study where concepts and principals for a classification system were established. The review of different systems was central in the pre-study. Concepts and principals established in the pre-study were important in later work on details, avoiding distractions and the risk of departing into wrong directions.

The source information was analysed and discussed during bi-weekly meetings between the author group or subgroups addressing specific subjects. During this process, a webinar was also arranged to present the concept and receive comments to the continued work from industry and public stakeholders within aquaculture. The author group also organised meetings and discussions with technical software systems specialists for the industry (e.g., FishTalk, Mercatus).

Communication and interaction with the project owner AquaCloud were a central part of the project, combining interaction with the industry and at the same time maintaining the integrity of the work as a research project.

3. Results

3.1. The purpose of a system for monitoring mortality and losses

The new technical digital solutions have made collection of data potentially unlimited in volume, including in the salmon industry. However, data collection must have a purpose, being useful and cost effective, and transform data into knowledge and better decisions within both industry and authorities (Osmundsen, Almklov, & Tveterås, 2017; Turnbull et al., 2011).

Monitoring mortality and losses must – in our opinion - have its overriding purpose in reducing losses and mortality in aquaculture, and consequently primarily as a tool for the animal owner and the responsibility for the animals under his/her care.

A modernized monitoring system must be able to both classify and quantify causes of death and other losses and needs to move from current focus on the qualitative results of diagnosis from individual animals to truly populations-based diagnosis and management. A system for industrial salmon farming must be dynamic in mode to handle changes in disease prevalence as well as to solve specific local and geographical needs. The system must serve changes in the needs of users and for all levels in the production chain, from the production site to corporate management. Furthermore, the system must be designed so that there is a correspondence between needs, ambitions, and purposes on the one hand, and the available resources in development and operation of the system on the other hand.

A key factor is the adoption of a standardized classification of causes, that would further allow comparisons between companies, regions and farming countries, and allow anonymized data to be made available for secondary users such as the supplier industry, governmental bodies, research, governmental bodies, NGO`s etc.

Software solutions for systems for effective input of data and data management for users at all levels are important for the compliance to a system. This requires development of the user experience (UX), application programming interfaces (API) and analytical tools at all levels. Currently, there are software companies supplying these services to the aquacultural industry. A standardized system for monitoring mortality and losses need to rely on- and comply with these systems and their technical solutions.

A system for quantifying the causes of loss and death need be a tool for industrial improvement and sustainable development, allocating resources to the area of most benefit. It must be possible to monitor trends and a tool for learning, better health management and reduced mortality and losses.

3.2. Causality as the principal criteria for classification

Aquaculture and especially salmon farming are industrial farming of animals in large populations. There is limited or no handling or treatment of individual fish, except euthanasia of moribund individual fish. Principally, classification of disease may have two principal approaches, being either a description of pathology (pathogenesis of disease) or describing the causality of disease. In human medicine, pet animals and part of terrestrial farming, like the dairy industry, a major part of health resources are used on treating the sick individuals. To effectively treat diseases, in depth knowledge of the pathogenesis of disease is required, in humans this goes down to ease mechanisms/ palliate treatment of death in final stages of life. Understanding and describing pathogenesis is further key elements in research on the treatment to improve the treatment and cure of disease in individuals. However, even the strong focus on treating individuals in humans the ICD use causality as the principle of monitoring mortality and morbidity (Brooke et al., 2017; Brooks & Reed, 2015; World Health Organization, 1979, 2019).

Health Management of salmon farming is largely about understanding and managing the underlying causes of mortality and losses with associated risk factors and contributing causes. In the handling of, or preferably preventing diseases, the focus needs to be on understanding the populations. At the same time fish welfare must be maintained at the fish level, meaning either euthanasia of individual fish, treatment of cages, or emergency harvest of cages. The complex process or "Train of events leading to death" (https://stats.oecd.org/glossary/detail.asp?ID=6325) is described in Figure 1. An understanding of this process and the use of universal glossary is important for systematic work on reducing mortality and losses.

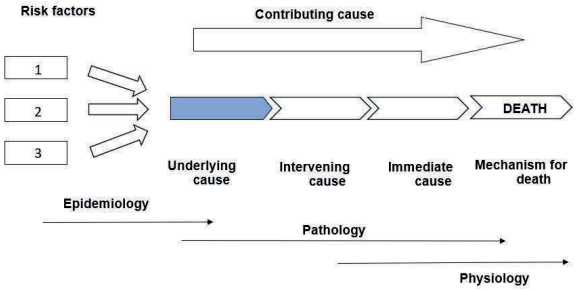

Figure 1. "The train of events leading to death" described as a combination of causality and pathogenesis. The underlying cause and its associated risk factors are proposed as the principal criteria 'of classifying mortality and morbidity and as a basis for health management in aquaculture.

Definition of the underlying cause of mortality of farmed salmon is suggested as follows:

> "The underlying cause of mortality, herein the disease that started the chain- reaction leading directly to death, or the external circumstances causing the deadly trauma, or the environmental condition causing the mortality, or the physiological state of the fish making it incompatible living in the surrounding environment."

The intervening cause, the immediate cause and the mechanism for death explain effects of disease rather than causes of disease (Figure 1). These effects of disease are of importance in diagnostics and in understanding pathogenesis of disease but is of less importance in causality of disease and in prevention of mortality and losses.

3.3. Hierarchical structure of the code system

A structure of the code in a useful hierarchy will enhance both the registration into the system and the retrieving and use of data out of a system. We propose the system to consist of three levels consisting of 6 major categories at the top level, currently 26 subcategories at level 2 and unlimited causes at level 3 (the underlying cause) (Figure 2). In the practical life (of fish farming) the need for underlying causes at level 3 is restricted, while in research or laboratory work there is a need for more detailed codes.

The recording of mortality cause is suggested to potentially take place at any level, for instance a suspicion of infectious disease can be placed at level 1, a suspicion of a viral diseases at level 2 and the specific underlying viral disease at level 3. The current software systems allow for the recording

of mortality due to a suspected cause, for later change of code or move down in the hierarchy when a lab-diagnosis is received, and investigations are complete.

The hierarchy will also be useful for a standard aggregation of data in statistics, reports etc. at any level (Figure 2). For the primary user (the fish farming company) this may be aggregating information at a site, region or in total for the company. Further, if having a unique code, it will be possible to aggregate data between regions or nationally for understanding mortality, studies, and resource allocation in health management.

For each underlying cause of death there will be an unlimited number of clinical signs, pathological findings, environmental observations etc. These will not be unique to a single underlying cause and will thereby not fit in to a hierarchical classification system. This information, however, is vital when investigating diseases and diagnostics in the population performed by the fish health professionals associated with the farm. And the diagnoses given by the fish health personnel are often equivalent to a cause of death in events of increased mortality. Hence, all this information is important in fish health management, the key element is how the data is structured and utilized.

It is crucial that the assignment of mortality will follow the fish group production data all the way through the production cycle. The same apply for possible risk factors, this enables life history analysis both as descriptive statistics and in methodological correct methods in risk factor analysis (Aunsmo 2009).

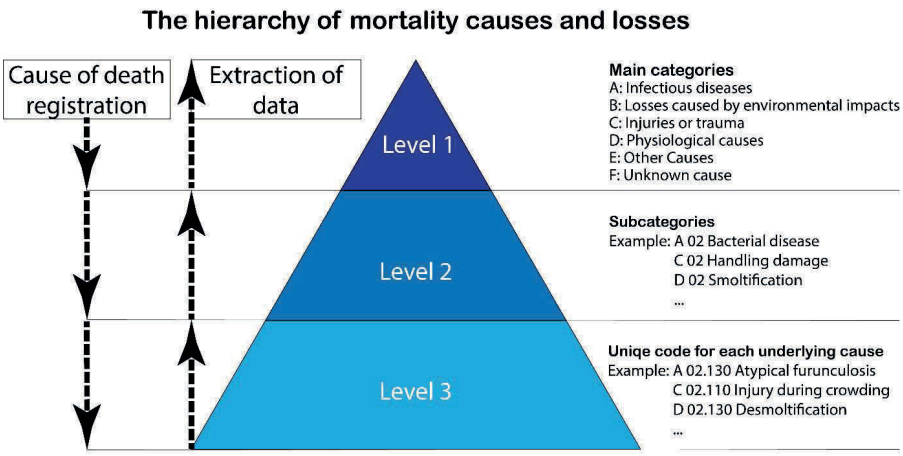

Figure 2. The suggested hierarchy of the code where both registration and reporting can take place at any level. The underlying causes are unique codes at level 3 and are aggregated at one sub-category only at level 2 and at one main category at level 1.

3.4. Expanding monitoring into other area of losses

We propose the development of a code that do not only cover the monitoring of cause-specific mortality, but a code that cover all causes ("morbidity") of losses in aquaculture. Illustrated in Figure 3 this includes individual fish effects in areas such as euthanasia, emergency harvest, escapees, quality downgrading at harvest, fish discard at harvest, and the population effects of feed conversion and growth. The effects of biology can further be combined with specific expenditures of disease and costs of prevention thereby making analysis of the cost of disease and finally cost-benefit analysis of preventing disease (Aunsmo et al., 2010; Pettersen et al., 2015). The different areas of losses are available for recording in current software-systems, however without a systematic code for the underlying causes total effects and costs cannot be summarized.

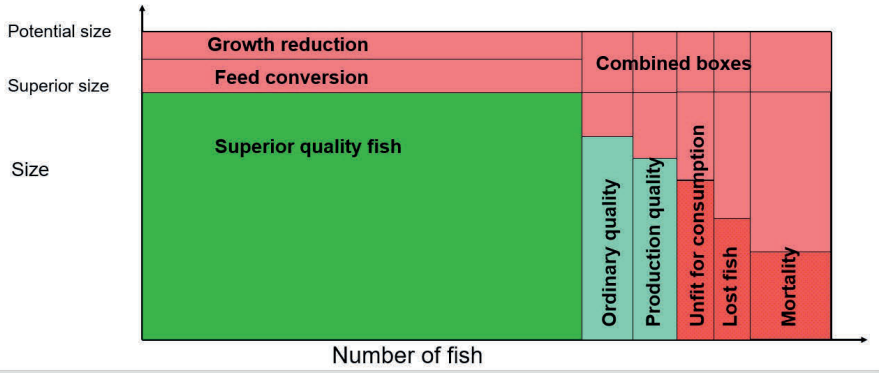

Figure 3. The biologic Production-Loss Model bPLM (Aunsmo, 2009) describing areas of losses in aquaculture where mortality and morbidity can be qualitatively (underlying causes) and quantitatively (numbers and biomass) monitored. The colours green and red indicate the severity of losses. Dark green is the part of production which is slaughtered and classified as superior quality and light green when fish is slaughtered with reduced quality. Light red indicates areas where biomass is lost in the production in terms of reduced growth and dark red where fish is lost in the production-reducing the number of fish reaching slaughter.

3.5. The principles of the proposed Code

The most recent version of the proposed full code is available as Supplement 1 to this paper.

The Code is suggested to consist of an alphanumeric code with an unlimited room for expansion. The specific code at level three is unique and will describe the level 1 category with a letter (A-F), the

following two numbers are level 2, subcategory (01-05), and the final three digits are the unique underlying cause at level 3 (Figure 2). Pancreas disease with SAV3 is thus registered with number A 01.127. All numbers ending with 0 denote group non-specific mortality/ losses at either level 1 or level 2. Not all mortalities or losses can be classified with necessary quality. We suggest that all mortalities or losses with low certainty of the underlying causes are registered as unknown. Under the main category "Unknown" (Letter F) we further suggest placing syndromes with unknown causality.

Level 3 causes should have a unique name and code, and where the name should denote the underlying cause. It will be possible to use both national and company specific names, as long as the code and definitions are unique.

To reduce faulty entry into the system each code is restricted to be only allowed in a logic area, in the correct life stage and for the correct species. Mechanical damage of harvested fish at the slaughter line cannot be a cause of death and precocious male cannot be cause for downgrading etc.

The software systems currently allow the individual company to restrict number of causes available at the specific production sites. This will further reduce risk for faulty registrations, and where the company list may easily be expanded if required.

3.6. Data quality and interactions with fish health professionals

The quality of recordings in a database of underlying causes for mortality and losses can be described with sensitivity and specificity of the investigations of mortality and losses when applied to populations (Maude & Ross, 1997; Serina et al., 2016). The sensitivity and specificity will be unique for each assignment of underlying cause (Murray, Lozano, Flaxman, Vahdatpour, & Lopez, 2011; Soofi et al., 2015). Since the classification of mortality into underlying causes are in place in most Norwegian salmon companies a standardized system will not start from zero, but a reasonable high level. Existing fish health services, a good standard of diagnostic laboratories and regulations requiring the investigation into increased mortality will contribute to quality. The interaction with professionals in many disciplines are important in improving the assignment of causality in other areas than infectious diseases, such as environmental and traumata (Figure 4).

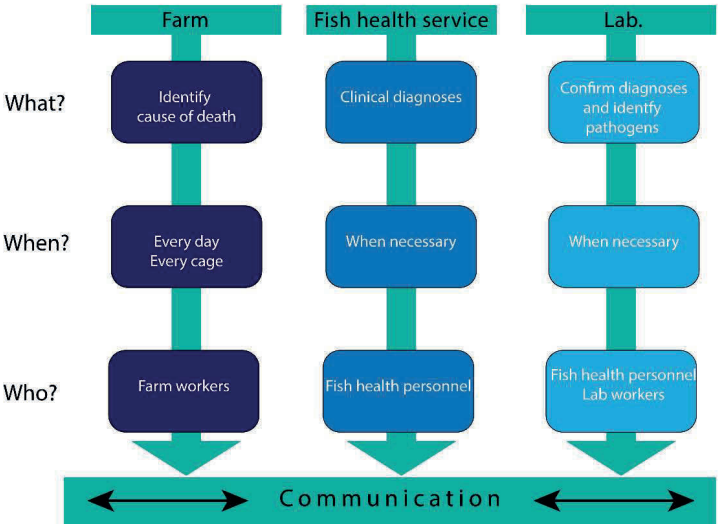

Figure 4. Principal description of the interaction between the operators at the farm, fish health services and the use of diagnostic laboratories. This interaction is of high importance in enhancing quality of registrations.

4. Discussion

Salmon farming is a young industry with many challenges to be addressed on the way to become a predictable and sustainable industry. To solve these challenges, we argue that monitoring cause-specific mortality and losses and secondly target resources in management, research and regulations towards these challenges will be an efficient and cost-effective use of limited resources.

4.1. Analogies and differences to monitoring cause-specific mortality in human medicine

In human health and in some parts of veterinary medicine it is a huge demand on treating individuals. For effective treatment it is necessary to understand the development and pathology of the disease for effectively monitor and treat the individual through the disease stages. This issue is not relevant in aquaculture where the only individual treatment is the humane culling of seriously ill animals. In all other aspects the treatment or handling of the population is the only option, and where the key element should be to prevent disease.

A major challenge in establishing the underlying cause of death is to separate it from the immediate cause of death (Brooke et al., 2017; Brooks & Reed, 2015; Ellis et al., 2012). An example is bacterial

ulcers where bacterial infection ultimately caused death but could not have occurred without the initial mechanical trauma (Aunsmo et al., 2008). The bacteria are possible to diagnose in a laboratory while the mechanical trauma is more diffuse to diagnose. This paradox easily leads to an overfocus of intermediate causes of pathology and death, losing the focus on underlying causes. Our concern is that focus on pathology and reduced fish welfare takes attention away from targeted interventions preventing the underlying causes to occur.

As mortality monitoring should stimulate effective interventions to remediate any dissatisfactory situation, the mortality recoding will add to the quantitative information if a qualitative cause of death is added. If the primary use of the data for the animal owner is optimized, secondary use of anonymized data – without reducing the data quality - for research and for analysis by the supplier industry, government is more easily facilitated. We also consider information on cause- specific mortality of interest for the public, where the salmon industry needs to use well established methods and be transparent to achieve a continuous acceptance to operate in the commons. Fish farmers operating in sea-based sites are utilizing resources and creating livelihood not only for the specific company, but also locally and regionally. In some countries the salmon industry also has become a large part of the nation's export values. In this respect the benefits of reducing losses in the salmon industry is way beyond the interests of the industry itself.

The alternative to an industry motivated classification system will be a regulatory motivated system, that is partly the case today. In a regulatory motivated system, we consider it more likely that it will be more focused on describing status rather than have a focus on prevention, with in-detail description of fish welfare hypotheses and detailed descriptions of pathology serving the as basis for decisions by authorities and creating unlimited databases for descriptive research projects. If a system mainly becomes academic in nature, we are afraid it will lose anchoring in the industry and become research oriented. We consider such an approach to have less or limited power in prevention of mortality and losses.

4.2. Fish welfare monitoring and improvement of fish health

It has been argued that monitoring mortality and cause- specific mortality is not a good indicator of fish welfare for the individual fish since it is to late – it is already dead (Ellis et al., 2012). On the contrary, we will argue that cause- specific mortality is a very good indicator of welfare at the population level, both quantitatively and qualitatively. Mortality is a very sensitive indicator where small changes in the environment for the fish will cause changes in mortality in populations of 150 000 individuals in the large salmon cages. Further it is specific in character, where well-developed diagnostics can reveal the underlying cause of mortality. We will further argue that cause-

specific mortality also is a very efficient indicator for directing limited resources in the direction of maximum effect of several outputs, including fish welfare. Developing the methods for monitoring underlying causes of death and losses may in this respect be of an efficient way of improving fish welfare. Current practices of describing fish welfare by monitoring effects of problems as pathology and other proxies gives in comparison good description of fish welfare proxies (Noble et al., 2018), but not being based on causality they offer less insight in effective prevention. Focus on intervening or immediate causes of death will lead to solving symptoms and effects of disease and not disease prevention.

The focus on underlying causes will also contribute to reducing risk later in the same population and prevent risk in new populations. Included is the option of culling sick or moribund individuals to reduce risk later in production. Also, whole cages may be removed to reduce risk later in production. By using causality as a principle, it may favour predictions on future happenings compared to using pathological descriptions.

4.3. Management use in aquaculture

The proposed change towards cause-specific mortality recording as a part of the management of the farm will give the farmer a novel, real time insight in the underlying problems, and improve the decision-making process towards a more sustainable production. The recording of mortality causes is based on all sources of information surrounding the production, both temporal and spatial. In this way the results from descriptive use of mortality can be used straight into the management process, nearly without translation. As an example, heart failure diagnosed with cardio myopathy syndrome (CMS) is diagnosed in three farms (A-C). This is a common diagnosis and cause of death in the later part of the marine phase in many farms (Bang Jensen, Mårtensson, & Kristoffersen, 2020; Brun et al., 2003). However, even if a farm has a diagnosis of CMS, the mortality cause can vary – both in time and between farms. Farm A has a continuously elevated mortality, and this is identified caused by CMS. Farm B has increased mortality because of a rough mechanical louse treatment, and the right causal cause would be lice treatment rather than CMS. In farm C mortality increased due to bad weather and strong currents and the causal cause would be strong current. For farmers to manage such increased mortality events, the different causes of death are important. Managing just from plain disease diagnoses would need to include a risk-factor analyses and a lot of data-input to obtain the same result as a mortality recording based on causality (i.e., lice treatment and harsh weather, in addition to CMS, in this case). Therefore, a mortality monitoring system based on causality would make the farmer acquire insight to the challenges in the production rapidly and simpler compared to thorough investigations of risk factors.

In order to work with this system, the company must agree on which level of accuracy they would like to operate under. Because the accuracy strongly depends on the effort the personal working at the farm performs in assigning the cause specific mortality to the dead fish from each cage each day. The mindset in how the system is going to work and improve the farming need to be implemented within the company.

Systematic registration of the underlying cause of death can be summed on a population (cage, farm, company etc.) and generate overview of the most common cause of death for that population within a chosen time frame. This has the potential to improve the farming operations, including health management, and gives the farmer a tool to more precise allocate the recourses towards to the field where they give the highest return both monetary and in terms of fish health

4.4. The underlying cause for use in descriptive statistics and risk factor analysis

By having specific codes for the underlying causes between companies it will be possible aggregate data for cause- specific mortality and losses in regions and nationally. The hierarchical structure makes it also possible to easily aggregate data in specified main categories and subcategories. This will add specific numbers into causes of mortality at a national level where today only crude number are reported while specific causes are based on interviews (Sommerset et al., 2021).

In both descriptive studies and in risk factor analysis it will also be possible to use data from several companies (Persson et al., 2021; Sviland Walde, Bang Jensen, Pettersen, & Stormoen, 2021). This do also allow studies on sources of variation and risk factors for downgrading of salmon like spinal deformities (Aunsmo et al., 2009). Cause-specific studies on both mortality and morbidity is the standard in human medicine, and necessary for detailed deciphering risk factors and sources of variation of underlying causes and losses in the complex production system of salmon farming.

4.5. Sensitivity and specificity

In salmon farming, autopsy and thorough investigation of individual fish to decide the cause of death would not be manageable in daily operation at the farm. Verbal autopsy, as a method for use in assigning mortality causes in human medicine without present medical professionals, are therefore of interest. And since the studies performed show that estimates of prevalence based on VA are considered adequate, this result is important also for salmon farming (Hernández et al., 2011; Murray et al., 2014; Soleman et al., 2006). In salmon farming assigning cause specific mortality will be conducted by the personnel working at the farm and may be considered less accurate than assessed by fish health personnel. However, from the studies in humans this suggest that results also from methods less accurate than autopsy would be acceptable to use and give valid results on population level (Quigley, Chandramohan, & Rodrigues, 1999; Serina et al., 2016; Soofi et al., 2015).

Mortality are also very often episode based, where one cause is a major factor causing mortality for a limited time period (Persson et al., 2021). This typically related to handling of fish, environmental impact, or infectious diseases. Such events are relatively easy to identify, when combining the knowledge from the operator at the farm and investigations from fish health personnel, to establish the true cause of death. The challenge is when the mortality is increasing slowly or are just marginally increased but over time. This would represent a situation where establishing a cause of death would be more difficult. However, this is when the use of "undefined" and "unknown" cause of death is advantageous. This helps reduce the number of false positive assignments and also increase the specificity of the system as a whole. When the mortality is accumulated on unknown causes it will automatically trigger further actions into investigating and reveal the underlying cause of death. Furthermore, using the categories undefined or unknown leaves the ranking of mortality causes unbiased. Thus, the use of unknown cause of death and unspecified mortality at a higher level in the hierarchy is important tools in a classification system. The use "unknown cause" should be accepted both as tool to improve quality of the database and as a tool to monitor the diagnostic effort of a company. Falsely low levels of "unknown causes" will impair quality of the databases, with the risk of assigning resources to non-existing problems and loosing focus on the real challenges.

Comparable to VA in human medicine the motivation for monitoring cause- specific mortality is to provide population-level mortality statistics, i.e. Cause-Specific Mortality Fractions (CSMF) and not cause of death for individuals (Serina et al., 2016).

Specific studies and datasets are used for developing VA-algorithms for deriving to underlying cause of death in human medicine. These specific studies serve the ICD to verify and develop the system. Detailed pathological findings and history at time of death belongs to the journal system of Fish health professionals as tools and recordings for the purpose of deriving populations diagnosis. Observations from the Fish Farmer on daily registrations should also be available for Fish Health Professionals in their diagnostic work.

5. Future development

The majority of mortality in salmon farming comes as episodes that are limited in time, indicating that most episodes have one dominant underlying cause (Aunsmo et al., 2008; Bang Jensen, Qviller, et al., 2020; Persson et al., 2021). The industry also has a well-developed diagnostic machinery with fish health services, laboratories etc. giving good and reliable diagnostic support. This makes it promising to be able to develop algorithms for ascertain underlying cause- of depth based on all available information, similar to VA in human medicine (Lozano et al., 2011; Murray et al., 2014). Such system should have inbuilt "levels of certainty" where new and unresolved mortality should call for more diagnostic work. Machine vision both in automatic mortality handling and filming live fish

combined with artificial intelligence may be useful methods in developing useful algorithms for a verbal autopsy analogue in aquaculture. Sensor information on environmental parameters and also knowledge about fish handling is all useful information for algorithms ascertaining the underlying cause of death.

In further development of software, it should be possible to connect all areas of losses and thereby be able to estimate the total effects of each of the underlying causes on production, fish welfare, economy, carbon footprint etc. By working systematic resources can be allocated to be most profitable area – using the equimarginal principle (Dijkhuizen & Morris, 1997).

6. Conclusion

Work on reducing mortality and losses in aquaculture needs to be based on prevention and where the underlying cause is proposed as the principal variable to monitor and improve. Enabling the fish farmer in making efficient decisions and improvement is considered essential in reducing mortality. Establishing a uniform code for classification of mortality and losses will enhance systematic work in monitoring, resource allocation and health management in aquaculture.

Appendices

1. The Code (level 3)

7. References

Akvakulturdriftsforskriften. (2008). Forskrift om drift av akvakulturanlegg. Retrieved from https://lovdata.no/forskrift/2008-06-17-822/

Animalia. (2021). Kodeliste for husdyrsjukdommer. Retrieved from https://www.animalia.no/no/Dyr/dyrehelse/kodelister/

Aunsmo, A. (2009). *Health related losses in sea farmed Atlantic salmon-quantification, risk factors and economic impact.* (Philosophiae Doctor). Oslo.

Aunsmo, A., Bruheim, T., Sandberg, M., Skjerve, E., Romstad, S., & Larssen, R. B. (2008). Methods for investigating patterns of mortality and quantifying cause-specific mortality in sea-farmed Atlantic salmon Salmo salar. *Diseases of Aquatic Organisms, 81*(2), 99-107. doi:10.3354/dao01954

Aunsmo, A., Valle, P. S., Sandberg, M., Midtlyng, P. J., & Bruheim, T. (2010). Stochastic modelling of direct costs of pancreas disease (PD) in Norwegian farmed Atlantic salmon (Salmo salar L.). *Preventive Veterinary Medicine, 93*(2-3), 233-241. doi:10.1016/j.prevetmed.2009.10.001

Aunsmo, A., Øvretveit, S., Breck, O., Valle, P. S., Larssen, R. B., & Sandberg, M. (2009). Modelling sources of variation and risk factors for spinal deformity in farmed Atlantic salmon using hierarchical- and cross-classified multilevel models. *Preventive Veterinary Medicine, 90*(1), 137-145. doi:10.1016/j.prevetmed.2009.03.015

Bang Jensen, B., Mårtensson, A., & Kristoffersen, A. B. (2020). Estimating risk factors for the daily risk of developing clinical cardiomyopathy syndrome (CMS) on a fishgroup level. *Preventive Veterinary Medicine, 175*, 104852. doi:10.1016/j.prevetmed.2019.104852

Bang Jensen, B., Qviller, L., & Toft, N. (2020). Spatio-temporal variations in mortality during the seawater production phase of Atlantic salmon (Salmo salar) in Norway. *Journal of Fish Diseases, 43*(4), 445-457. doi:10.1111/jfd.13142

Brooke, H. L., Talbäck, M., Hörnblad, J., Johansson, L. A., Ludvigsson, J. F., Druid, H., . . . Ljung, R. (2017). The Swedish cause of death register. *European Journal of Epidemiology, 32*(9), 765-773. doi:10.1007/s10654-017-0316-1

Brooks, E. G., & Reed, K. D. (2015). Principles and Pitfalls: a Guide to Death Certification. *Clinical medicine & research, 13*(2), 74-84. doi:10.3121/cmr.2015.1276

Brun, E., Poppe, T., Skrudland, A., & Jarp, J. (2003). Cardiomyopathy syndrome in farmed Atlantic salmon Salmo salar: occurrence and direct financial losses for Norwegian aquaculture. *Diseases of Aquatic Organisms, 56*(3), 241-247. doi:10.3354/dao056241

Crockford, T., Menzies, F., McLoughlin, M., Wheatley, S., & Goodall, E. (1999). Aspects of the epizootiology of pancreas disease in farmed Atlantic salmon Salmo salar in Ireland. *Diseases of Aquatic Organisms, 36*(2), 113-119.

Dijkhuizen, A. A., & Morris, R. S. (1997). *Animal Health Economics: principles and applications.* Sidney: University of Sydney.

Directorate of Fisheries. (2021). *Lønnsomhetsundersøkelse for laks og regnbueørret: matfiskproduksjon*. Retrieved from Directorate of fisheries Norway: https://www.fiskeridir.no/Akvakultur/Tall-og-analyse/Loennsomhetsundersoekelse-for-laks-og-regnbueoerret/Matfiskproduksjon-laks-og-regnbueoerret

Directorate of Fisheries. (2022). Norwegian aquaculture statistics. Retrieved from https://www.fiskeridir.no/Akvakultur/Tall-og-analyse/Akvakulturstatistikk-tidsserier/Laks-regnbueoerret-og-oerret

Elanco. (2020). *Manual clasificaión de mortlidad de peces, version 3*. Retrieved from Puerto Varas, Chile: https://www.elancoaquaglobal.com/cl/es/news#reports

Ellis, T., Berrill, I., Lines, J., Turnbull, J. F., & Knowles, T. G. (2012). Mortality and fish welfare. *Fish Physiology and Biochemistry, 38*(1), 189-199. doi:10.1007/s10695-011-9547-3

Faroese Food and Veterinary Authority. (2019). Executive order no 80 of 14 June 2019 on the establishment of - and disease prevention in aquaculture facilities. Retrieved from

https://www2.hfs.fo/webcenter/ShowProperty?nodeId=%2Fhfs2-cs%2FHFS059686%2F%2FidcPrimaryFile&revision=latestreleased
Faroese seafood. (2022, 3 May). Aquaculture – Legislation and Management. Retrieved from https://www.faroeseseafood.com/fishery-aquaculture/aquaculture-legislation-and-management/
Forskrift om sykdom hos dyr. (2015). Forskrift om varsel og melding om sjukdom hos dyr. Retrieved from https://lovdata.no/dokument/SF/forskrift/2014-12-19-1841
Hernández, B., Ramírez-Villalobos, D., Romero, M., Gómez, S., Atkinson, C., & Lozano, R. (2011). Assessing quality of medical death certification: Concordance between gold standard diagnosis and underlying cause of death in selected Mexican hospitals. *Population Health Metrics, 9*(1), 38. doi:10.1186/1478-7954-9-38
Icelandic Food and Veterinary Authority, M. (2020). Article 56, regulation on aquaculture, no. 540/2020.
Iversen, A., Asche, F., Hermansen, Ø., & Nystøyl, R. (2020). Production cost and competitiveness in major salmon farming countries 2003–2018. *Aquaculture, 522*, 735089. doi:10.1016/j.aquaculture.2020.735089
Karreman, G. A. (1991). *Co-operative assessment of salmonid health record of performance program for the fin fish industry in British Columbia*. Retrieved from Pacific marine veterinary services.
Kilburn, R., Murray, A. G., Hall, M., Bruno, D. W., Cockerill, D., & Raynard, R. S. (2012). Analysis of a company's production data to describe the epidemiology and persistence of pancreas disease in Atlantic salmon (Salmo salar L.) farms off Western Scotland. *Aquaculture, 368-369*, 89-94. doi:10.1016/j.aquaculture.2012.09.004
Knibbs, G. H. (1929). The International Classification of Disease and Causes of Death and its Revision. *Medical Journal of Australia, 1*, 2-12. Retrieved from https://www.cabdirect.org/cabdirect/abstract/19292701569
Lozano, R., Lopez, A. D., Atkinson, C., Naghavi, M., Flaxman, A. D., & Murray, C. J. L. (2011). Performance of physician-certified verbal autopsies: multisite validation study using clinical diagnostic gold standards. *Population Health Metrics, 9*(1), 32. doi:10.1186/1478-7954-9-32
Marine Scotland. (2009). Aquatic animal health (Scotland) Regulations 2009. Retrieved from https://www.legislation.gov.uk/ssi/2009/85/contents
Marine Scotland. (2021). Scottish fish farm production survey data. Retrieved from www.doi.org/10.7489/1918-1
Marinhelse. (2018). *Veileder i dødfiskkategorisering*. Tromsø, Norway: MarinHelse AS.
Maude, G. H., & Ross, D. A. (1997). The effect of different sensitivity, specificity and cause-specific mortality fractions on the estimation of differences in cause-specific mortality rates in children from studies using verbal autopsies. *International Journal of Epidemiology, 26*(5), 1097-1106. doi:10.1093/ije/26.5.1097
Menzies, F. D., McLoughlin, M., Wheatley, S., & Goodall, E. A. (1996). Development of a computerized information retrieval system for Atlantic salmon, Salmo salar L., production. *Aquaculture Research, 27*, 183-190.
Murray, C. J. L., Lozano, R., Flaxman, A. D., Serina, P., Phillips, D., Stewart, A., . . . Lopez, A. D. (2014). Using verbal autopsy to measure causes of death: the comparative performance of existing methods. *BMC Medicine, 12*(1), 5. doi:10.1186/1741-7015-12-5
Murray, C. J. L., Lozano, R., Flaxman, A. D., Vahdatpour, A., & Lopez, A. D. (2011). Robust metrics for assessing the performance of different verbal autopsy cause assignment methods in validation studies. *Population Health Metrics, 9*(1), 28. doi:10.1186/1478-7954-9-28
Naghavi, M., Abajobir, A. A., Abbafati, C., Abbas, K. M., Abd-Allah, F., Abera, S. F., . . . Murray, C. J. L. (2017). Global, regional, and national age-sex specific mortality for 264 causes of death, 1980–2016: a systematic analysis for the Global Burden of Disease Study 2016. *The Lancet, 390*(10100), 1151-1210. doi:https://doi.org/10.1016/S0140-6736(17)32152-9

Noble, C., Gismervik, K., Iversen, M. H., Kolarevic, J., Nilsson, J., Stien, L. H., & Turnbull, J. F. (2018). *Welfare Indicators for farmed Atlantic salmon: Tools for assessing fish welfare*. Retrieved from NOFIMA: http://www.nofima.no/fishwell/english

Osmundsen, T. C., Almklov, P., & Tveterås, R. (2017). Fish farmers and regulators coping with the wickedness of aquaculture. *Aquaculture Economics & Management, 21*(1), 163-183. doi:10.1080/13657305.2017.1262476

Persson, D., Nødtvedt, A., Aunsmo, A., & Stormoen, M. (2021). Analysing mortality patterns in salmon farming using daily cage registrations. *Journal of Fish Diseases, 00*, 1-13. doi:10.1111/jfd.13560

Pettersen, J. M., Rich, K. M., Jensen, B. B., & Aunsmo, A. (2015). The economic benefits of disease triggered early harvest: A case study of pancreas disease in farmed Atlantic salmon from Norway. *Preventive Veterinary Medicine, 121*(3-4), 314-324. doi:10.1016/j.prevetmed.2015.08.003

Pincinato, R. B. M., Asche, F., Bleie, H., Skrudland, A., & Stormoen, M. (2021). Factors influencing production loss in salmonid farming. *Aquaculture, 532*, 736034. doi:10.1016/j.aquaculture.2020.736034

Quigley, M. A., Chandramohan, D., & Rodrigues, L. C. (1999). Diagnostic accuracy of physician review, expert algorithms and data-derived algorithms in adult verbal autopsies. *International Journal of Epidemiology, 28*(6), 1081-1087. doi:10.1093/ije/28.6.1081

Serina, P., Riley, I., Hernandez, B., Flaxman, A. D., Praveen, D., Tallo, V., . . . Lopez, A. D. (2016). The paradox of verbal autopsy in cause of death assignment: symptom question unreliability but predictive accuracy. *Population Health Metrics, 14*(1), 41. doi:10.1186/s12963-016-0104-2

Sernapesca. (1995). Establece reglamento para la entrega de información de pesca y acuicultura y la acreditación de origen deja sin efecto decreto nº 464, de 1995 (In spanish: Established regulations for the reporting of fisheries and aquaculture information and accreditation of origin leave without effect decree nº 464, of 1995). Retrieved from http://www.sernapesca.cl/sites/default/files/2020_0101_reglamento.pdf

Sernapesca. (2012). Resolucion exenta n 1468: Aprueba programa sanitario general de manejo de mortalidades y su sistema de clasificación estandarizado conforme a categorías preestablecidas. (In spanish: Approved general health program for managing mortality and its standardized classification system according to pre-established categories). Retrieved from http://www.sernapesca.cl/sites/default/files/resex_1468_2012.pdf

Sernapesca. (2021). *Informe sanitario de la salmonicultura en centros marinos 1° semestre 2021 (in spanish: Health report on salmon farming in marine centers, 1st semester 2021)*. Retrieved from Sernapesca: http://www.sernapesca.cl/sites/default/files/informe_sanitario_salmonicultura_en_centros_marinos_1_semestre_2021.pdf

Soares, S., Green, D. M., Turnbull, J. F., Crumlish, M., & Murray, A. G. (2011). A baseline method for benchmarking mortality losses in Atlantic salmon (Salmo salar) production. *Aquaculture, 314*(1), 7-12. doi:10.1016/j.aquaculture.2011.01.029

Soares, S., Murray, A. G., Crumlish, M., Turnbull, J. F., & Green, D. M. (2012). Evaluating abnormal mortality as an indicator of disease presence in the Atlantic salmon industry using the receiver operating characteristic (ROC). *Aquaculture, 370-371*, 136-143. doi:10.1016/j.aquaculture.2012.10.004

Soares, S., Murray, A. G., Crumlish, M., Turnbull, J. F., & Green, D. M. (2013). Factors affecting variation in mortality of marine Atlantic salmon Salmo salar in Scotland. *Diseases of Aquatic Organisms, 103*(2), 101-109. doi:10.3354/dao02562

Soleman, N., Chandramohan, D., & Shibuya, K. (2006). Verbal autopsy: current practices and challenges. *Bulletin of the World Health Organization, 84*(3), 239-245. doi:10.2471/blt.05.027003

760 Sommerset, I., Bang Jensen, B., Bornø, B., Haukaas, A., & Brun, E. (2021). *Fish Health Report 2020*.
761 Retrieved from Norwegian Veterinary Institute: https://www.vetinst.no/en/reports-and-
762 publications/reports
763 Soofi, S. B., Ariff, S., Khan, U., Turab, A., Khan, G. N., Habib, A., . . . Bhutta, Z. A. (2015). Diagnostic
764 accuracy of WHO verbal autopsy tool for ascertaining causes of neonatal deaths in the urban
765 setting of Pakistan: a hospital-based prospective study. *BMC Pediatr, 15*, 144.
766 doi:10.1186/s12887-015-0450-4
767 Sviland Walde, C., Bang Jensen, B., Pettersen, J. M., & Stormoen, M. (2021). Estimating cage-level
768 mortality distributions following different delousing treatments of Atlantic salmon (salmo
769 salar) in Norway. *Journal of Fish Diseases, 44*(7). doi:10.1111/jfd.13348
770 Turnbull, J. F., Berrill, I. K., Green, D. M., Kaye, R., Morris, D., Murray, A. G., . . . Shinn, A. (2011).
771 Applied epidemiology with examples from UK aquaculture. *Aquaculture Research, 42*(s1), 21-
772 27. doi:10.1111/j.1365-2109.2010.02667.x
773 VetStat. (2021). Danish Veterinary and Food Administration. Retrieved from
774 https://www.foedevarestyrelsen.dk/Leksikon/Sider/VetStat.aspx
775 Wheatley, S. B., McLoughlin, M. F., Menzies, F. D., & Goodall, E. A. (1995). Site management factors
776 influencing mortality rates in Atlantic salmon (Salmo salar L.) during marine production.
777 *Aquaculture, 136*(3), 195-207. doi:10.1016/0044-8486(95)01058-0
778 World Health Organization. (1979). *Medical certification of cause of death, Instructions for physicians*
779 *on use of international form of medical certificate of cause of death*. Retrieved from World
780 Health Organization: https://apps.who.int/iris/handle/10665/40557
781 World Health Organization. (2016). *Verbal autopsy standards: The 2016 WHO verbal autopsy*
782 *instrument (V.1.5.3)*. Retrieved from World Health Organization:
783 https://www.who.int/publications/m/item/verbal-autopsy-standards-the-2016-who-verbal-
784 autopsy-instrument
785 World Health Organization. (2019). *ICD-11, International classification of diseases for mortality and*
786 *morbidity statistics*(11th Revision ed.). Retrieved from https://icd.who.int/en
787 World Health Organization. (2021a). *History of the development of the ICD*. Retrieved from World
788 Health Organization: https://www.who.int/publications/m/item/history-of-the-
789 development-of-the-icd
790 World Health Organization. (2021b). *World health statistics 2021: monitoring health for the SDGs,*
791 *sustainable development goals*. Retrieved from World Health Organization:
792 https://apps.who.int/iris/handle/10665/342703.

793

Level 1	Level 2	Level 3/code	Name	Short name
A			***A INFECTIOUS DISEASES***	
	A.00	A 00.100	**Infectious disease - not specified**	Infectious not spec
	A 01	A 01.100	**Viral disease - not specified**	Viral not spec
		A 01.110	Viral hemorragic septicamia (VHS)	VHS
		A 01.120	Infectious hematopoietic necrosis (IHN)	IHN
		A 01.130	Infectious salmon anemia (ISA)	ILA
		A 01.131	Avirulent ISA-virus (HPR0)	HPR0
		A 01.140	Pancreas disease (not specified)	PD
		A 01.142	Pancreas disease caused by SAV2	PD-SAV2
		A 01.143	Pancreas disease caused by SAV3	PD-SAV3
		A 01.150	Infectious pancreatic necrosis (IPN)	IPN
		A 01.160	Heart and skeletal muscle inflammation (HSMI)	HSMI
		A 01.170	Cardiomyopathy syndrome (CMS)	CMS
		A 01.180	Poxvirus gill disease (of salmon)	Gill pox
	A 02	A 02.100	**Bacterial disease not specified**	Bacterial not spec
		A 02.110	Bacterial kidney disease (BKD)	BKD
		A 02.120	Classical furuculosis	Furunc
		A 02.130	Atypical furunculosis	Atypical furunc
		A 02.140	Rainbow trout fry syndrome (RTFS)	RTFS
		A 02.150	Snout and head ulcers (Tenacibaculum spp).	Snout ulcers
		A 02.160	Winter ulcers caused by Moritella viscosa	Winter ulcers
		A 02.170	Wounds caused by not specified bacteria	Wounds
		A 02.180	Bacterial gill disease	BGD
		A 02.190	Fin rot /Saddleback disease	Fin Rot
		A 02.200	Piscirickettsiosis	SRS
		A 02.210	Generalised infection with Streptococcus spp	Strep
		A 02.220	Classical vibriosis caused by V. anguillarum/ ordalii	Vibriosis
		A 02.230	Coldwater vibriosis	CWV
		A 02.250	Enteric redmouth disease/ Disese caused by Yersinia ruckeri	ERM
		A 02.260	Pasteurellosis	Pasteurellosis
	A 03	A 03.100	**Parasitic disease not specified**	Parasite not spec
		A 03.110	Costiasis (Ichthyobodiasis)	Costiasis
		A 03.120	White spot disease	"Ich"
		A 03.130	Whirling disease	Whilring disease
		A 03.140	Skin trichodinosis	Trichodina
		A 03.150	Gyrodactylus salaris infection	G. salaris
		A 03.160	Tapeworm	Tapeworm
		A 03.170	Parvicapsula	Parvicapsula
		A 03.180	Salmon lice (L. salmonis)	Salmon lice
	A 04	A 04.100	**Fungal infection not specified**	Funghi not spec
		A 04.110	Saprolegniosis	Sapro
		A 04.120	Kidney fungus (Exophiala)	Exophiala
	A 05	A 05.100	**Amoebic disease not specified**	Amoeba not spec
		A 05.110	Amoebic gill disease	AGD
B			***B LOSSES CAUSED BY ENVIRONMENTAL IMPACTS***	
	B.00	B 00.100	**Environmental Disease not specified**	Environment not spec
	B 01	B 01.100	**Impact caused by natural environment not specified**	Natural environment not spec
		B 01.110	Storm and hurricane	Storm
		B 01.111	Extreme tide	Tide
		B 01.112	Extreme sea current	Current
		B 01.115	Flooding	Flood
		B 01.120	Hypoxia in natural waters	Hypoxia
		B 01.130	Gas supersaturation of water	Supersat
		B 01.135	Toxic algae	Toxic algae
		B 01.140	Extreme low water temperature	Low water temp
		B 01.141	Extreme high water temperature	High water temp
		B 01.150	Toxic jellyfish	Jellyfish

Level 1	Level 2	Level 3/code	Name	Short name
B	B 02	B 02.100	**Failure of environmental control not specified**	Control system failure
		B 02.110	Failure of water supply	Water supply
		B 02.120	Failure of oxygen supply	Oxygen supply
		B 02.130	CO2 poisoning	CO2
		B 02.140	Jumper	Jumper
		B 02.150	Nephrocalcinosis	Kidney calcinosis
		B.02.160	Gas bubble disease	Gas bubble
		B 02.170	Malformation induced by egg incubation temperature	Malformations (temp)
	B 03	B 03.100	**Intoxication not specified**	Intoxication not spec
		B 03.110	Copper poisoning	Cu++
		B 03.120	Iron precipitation on the gills	Fe++
		B 03.130	Aluminium poisoning	Al++
		B 03.140	Nitrite poisoning	NO2-
		B 03.150	Ammonia poisoning	NH4
		B 03.160	Hydrogen sulfide gas poisoning	H2S
		B 03.170	Chlorine poisoning	Chlorine
C			**C INJURIES (TRAUMA)**	
	C 00	C 00.100	**Injury unspecified/unknown cause**	Injury unknown/not spec
	C 01	C 01.100	**Predators not specified**	Predator not spec
		C 01.110	Bird not specified	Bird not spec
		C 01.120	Heron	Heron
		C 01.130	Cormorant	Cormorant
		C.01.200	Terestrial animals not specified	Land animals not spec
		C 01.210	Otter	Otter
		C 01.220	Seal	Seal
		C 01.230	Sea lion	Sea lion
		C 01.300	Lesions caused by intruders	Intruders
	C 02	C 02.100	**Handling damage not specified**	Handling not spec
		C 02.110	Injury during crowding	Crowding
		C 02.120	Injury caused by pumping and lifting equipment	Pumping
		C 02.130	Injury during vaccination	Vaccination
		C 02.140	Transport death and injury on wellboat	Boat transport
		C 02.150	Transport death and injury (tanks)	Tank transport
		C 02.160	Faulty technical equipment	Equipment
		C 02.170	Gill cover damage	Operculum
		C 02.180	Eye damage	Eye damage
	C 03	C 03.100	**Non-medical treatment unspecified**	Non-medical unspec
		C 03.110	Hydrolicer	Hydrolicer
		C 03.120	Thermolicer	Thermolicer
		C 03.130	Optilicer	Optilicer
		C 03.140	Skamik	Skamik
		C 03.150	Flatsetsund washer	FLS
		C 03.160	Wellfighter	Wellfighter
		C 03.170	Fresh water treatment	Freshwater
	C 04	C 04.100	**Medicinal treatment not specified**	Treatment not spec
		C 04.110	Overdose	Overdose
		C 04.120	Medical treatment in sea cage	Sea cage treatment
		C 04.130	Medical treatment in wellboat	Wellboat tretment
	C 05	C 05.100	**Side effect of medicinal treatent not specified**	Side-effect not spec
		C 05.110	Vaccination side effect (injection site)	Injection-site
		C 05.120	Indirect vaccination side effect (spinal deformity)	Indirect side-effect (spinal)
		C 05.130	Abdominal adhesions	Adhesions

Level 1	Level 2	Level 3/code	Name	Short name
			D PHYSIOLOGICAL CAUSES	
D	**D 00**	D 00.100	**Physiological maladaptation not specified**	Maladapation not spec
	D 01	D 01.100	**Embryo- and fry mortality not specified**	Egg and fry not spec
		D 01.110	White (coagulated) eggs	White egg
		D 01.120	Yolk sac deformity	Yolk sac
		D 01.130	Blue sac disease	Blue sac
	D 02	D 02.100	**Smoltification not specified**	Smolting not spec
		D 02.110	Haemorrhagic smolt syndrome (HSS)	HSS
		D 02.120	Incomplete smoltification	Incomplete smolting
		D 02.130	Desmoltification	Desmoltified
	D 03	D 03.100	**Incomplete physiological adaptation**	Not adapted physiologically
		D 03.110	Sexual maturation	Mature
		D 03.120	Precocious males	Precox
		D 03.130	Gastric dilatation in seawater reared Rainbow Trout	Gastric dilation
			E OTHER CAUSES	
E	**E 00**	E 00.100	**Other cause not specified**	Other cause not spec
	E 01	E 01.000	**Tumor**	Tumor
	E 02	E 02.000	**Disease caused by suspected nutritinal deficiency**	Suspected deficiency
		E 02.110	Short-tail	Short-tail
		E 02.120	Cataract	Cataract
		E 02.130	Vertebral deformity	Vertebral deform
		E 02.140	Malformation of head or jaws	Head or jaw deformity
		E 02.150	Aggression (eye, gill cover or dorsal fin damage)	Aggression
	E 03	E 03.100	**Euthanised after grading no disease or injury**	Grading without disease
		E 03.110	Euthanized - no sale	No sale
	E 04	E 04.000	**Sampling of fish without signs of disease**	Sampling, healthy
	E 05	E 05.100	**Quality downgrade after harvest -unspecified**	Downgrade
		E 05.110	Lost on the floor	Floor fish
		E 05.120	Improper cut (exsanuination, evisceration etc.)	Bad cut
		E 05.130	Bile color in abdomen	Bile discoloration
		E 05.140	Melanin in or on fillet	Melanin in/on fillet
		E 05.150	Red belly	Red belly
		E 05.160	Healed wounds (pigmented)	Scars
		E 05.170	Incomplete exsanguiation	Poorly bled
		E 05.180	Carcass damage (post mortem)	Squeezed
		E 05.190	Dead before slaughter	Dead before harvest
		E 05.200	Net damage	Net damage
		E 05.210	Abnormal color	Abnormal color
		E 05.220	Emaciated fish	Emaciated
		E 05.230	Extensive scale loss	Scale loss
	E 06	E 06.000	**Escapee not specified**	Escapee not spec
		E 06.110	Escapee at grading	Escapee at grading
			F UNKNOWN CAUSE	
F	**F 00**	F 00.100	Unknown cause of death	Unknown cause of death
	F 01	F 01.100	**Syndromes with unknown cause of death**	Syndromes
		F 01.110	Runts (emaciation) with unknown cause	Runts unknown cause
		F 01.120	Complex gill disease with unknown cause	CGD

Paper III

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ORIGINAL ARTICLE

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Variations in mucous cell numbers in gills of Atlantic salmon (*Salmo salar*) presmolt in commercial freshwater farms in Norway

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Abstract

Fish gills are heavily exposed to the external milieu and may react against irritants with different cellular responses. We describe variations in mucous cell counts in gills from healthy Atlantic salmon (*Salmo salar*) presmolts in five recirculating aquaculture system (RAS) farms and one flow-through farm. Based on certain criteria, mucous cells were histologically quantified in a defined lamellar region of the gills and the counts were analysed. Immunohistochemistry (IHC) was used to investigate epithelial responses. The median number of total mucous cells in the defined region was 59 per fish. Between the farms, the medians varied from 31 to 101 with the lowest in the flow-through farm. A regression model was fitted with "total mucous cells" as the dependent variable and with "fish length" and "fish farm" as independent variables. The proportion of variation in mucous cell counts explained by the model was twice as high when "fish farm" was included compared to only "fish length." IHC revealed proliferative responses in coherence with high mucous cell numbers. Conclusively, the variation in mucous cell counts depends on combined farm-related factors. Establishing a baseline for mucous cell counts is fundamental in the development of high-throughput monitoring programmes of gill health in farmed fish.

KEYWORDS

gill health, mucous cell, RAS, salmon

1 | INTRODUCTION

Traditionally, salmon (*Salmo salar*) smolt have been reared in land-based facilities using water from nearby rivers or lakes which flow through the production site. Flow-through farms (FT farms) depend on high water quality from the source, as means of regulating different water parameters are limited (Kristensen, Åtland, Rosten, Urke, & Rosseland, 2009). Recirculating aquaculture system (RAS) technology has developed rapidly over the last years and has been preferred in new production facilities for rearing of salmon in the freshwater stage. Because RAS technology allows close to 100% recirculation of water, it is possible to produce more fish with restricted resources (Dalsgaard et al., 2013). However, reuse of water demands efficient handling of accumulating waste products, such as particles, which

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must be removed. Additionally, the maintenance of pH levels and dissolved gases is imperative to ensure a stable environment for the fish (Dalsgaard et al., 2013; Hjeltnes et al., 2012; Kristensen et al., 2009).

Breaching of the biosecurity in RAS facilities is often critical. Accumulation of waste products can cause physical stress, which could result in reduced function of the gills (Awata, Tsuruta, Yada, Iguchi, & i., 2011; Bruton, 1985; Dahle et al., 2020). Because of their delicate structure and close contact with water, the gills are particularly vulnerable for mechanical damage and injuries caused by environmental factors (Rodger, 2007). The gills are vital not only for respiratory purposes, but also for osmoregulation, nitrogenous waste excretion, pH regulation and production of hormones (Evans, Piermarini, & Choe, 2005). In addition, the gill epithelium is an essential part of the immune system, acting as a physical and functional barrier against the outer environment (Gomez, Sunyer, & Salinas, 2013; Koppang, Kvellestad, & Fischer, 2015).

Studies of the human respiratory tract have shown that proliferation of mucous cells (or goblet cells) is one of the initial reactions towards exogenous irritants in the airways, with a resulting increase in mucus production (Rogers, 1994; Whitsett & Alenghat, 2015). Similar mechanisms are also present in the gills of fish (Gomez et al., 2013). Thus, mucous cell counts have been used to evaluate gill health in several experimental studies in salmonids (H. W. Ferguson, Morrison, Ostland, Lumsden, & Byrne, 1992; Roberts & Powell, 2003; Speare, Arsenault, MacNair, & Powell, 1997). A recent study on the histopathological responses involved in complex gill disease in farmed Atlantic salmon concluded that mucous cell hyperplasia was one of the most common pathological features (Gjessing et al., 2019). This corresponds well with the extensive mucus covering of diseased gills, a typical clinical sign of gill inflammation. Despite the well-known importance of mucous cell reactions in gills, there is scarce information on the variation of mucous cells between individual fish or possibly the variation between different fish populations. Thus, investigations addressing these questions are warranted. A baseline for mucous cell counts is fundamental in the development of a future high-throughput monitoring programme of gill health in farmed fish. This would be of special interest in RAS facilities, where gill health has been pinpointed as one of the critical concerns (Becke, Schumann, Steinhagen, Geist, & Brinker, 2018; Dahle et al., 2020; Hjeltnes et al., 2012).

In this cross-sectional study, the overall aim was to investigate the prevalence of mucous cells in presmolts from five different RAS facilities and one FT farm. This was approached through two objectives: first, to implement a counting method of mucous cells in salmon gills, and second, to describe the variations in mucous cell count in salmon and between different salmon production sites.

2 | MATERIALS AND METHODS

2.1 | Material

Gill samples from a total of 220 fish were collected from six different commercial land-based salmon farms on the western and northern coast of Norway from October 2018 to January 2019. Five of the farms were RAS-based (RAS I-V), and one was a traditional flow-through facility (FT I). Two samplings of 20 fish from two different tanks were conducted at each RAS farm (a total of 40 fish from each farm) with 14 days between the samplings. From the FT farm, only one sampling of 20 fish was carried out. All fish were reported to be healthy and without signs of clinical disease. The samplings were performed in the time period between vaccination and sea transfer.

2.2 | Sampling procedure

The fish were gently netted out from the tanks and killed by an overdose of sedation (Finquel© vet, Scan Aqua), in line with regulations of the Norwegian Directorate of Fisheries (Akvakultudriftsforskriften, 2008). Weight and length were recorded. The entire second gill arch on the left side was sampled and placed in 10% buffered formalin.

2.3 | Weight and length

Weight and length of the fish were recorded from all but one fish (from RAS III). In further calculations, the fish with the missing values was given the calculated mean weight and length from the nine other individuals from the same tank at the same sampling day.

2.4 | Water transparency

Water transparency was assessed by measuring the sight depth with a modified white Secchi disc of 15 cm in diameter in each tank at each sampling time point. The measured sight depth was divided by the tank depth to get the relative sight depth expressed in percentage of the tank depth to allow comparison of results across farms. If the bottom of the tank were visible, no measurement was performed, and the water transparency was put to 100%.

2.5 | Histological investigations

After fixation for minimum 48 hr, the gill arches were processed for histology and embedded in paraffin. All samples were embedded with identical tissue orientation. Sections (2 μm) were cut and stained with haematoxylin and eosin (HE) for histological investigation and periodic acid–Schiff (PAS) according to standard procedures for the detection of mucins (Bancroft & Gamble, 2008).

Immunohistochemical investigations were applied to investigate proliferation of cells (proliferative cell nuclear antigen [PCNA], dilution 1:5,000, Dako, DK-2600 Glostrup, Denmark) and the distribution of epithelial cells (pan-cytokeratin, clone AE1/AE3, dilution 1:50, Invitrogen, Thermo Fisher, MA, USA). Gills from the fish with the lowest (n = 3) and highest (n = 3) mucous cell counts, respectively, were chosen for IHC analysis (gills from six fish in total). The method has been described

in detail elsewhere (Bjørgen et al., 2018). Briefly, sections (4 μm) were cut and mounted on Superfrost+ slides (Mentzel, Braunschweig, Germany). The sections were de-paraffinized and autoclaved at 121 degrees Celsius for 10 min. The slides were treated with phenylhydrazine (0.05%; Sigma-Aldrich, St. Louis, MO, USA) at 37 degrees Celsius for 40 min to inhibit endogenous peroxidase. Non-specific binding in reactive sites was blocked with a solution of normal goat serum diluted in 5% bovine serum albumin/Tris-buffered saline (BSA/TBS). The slides were incubated with primary antibodies for 30 min in room temperature, washed three times in phosphate-buffered saline (PBS) and further incubated with the secondary antibody (Envision© System Kit; Dako, Glostrup, Denmark) for 30 min in room temperature. Red colour was evoked with 3-amino-9-ethylcarbazole (AEC) substrate incubated for 14 min. The slides were washed in distilled water and mounted using Aquatex® (Sigma-Aldrich) mounting medium. Negative controls were prepared with PBS instead of primary antibody.

2.6 | Mucous cell count

A counting method was established to investigate the prevalence of mucous cells in the lamellae of the gills in all groups (RAS I-V and FT I). Details on method development are described in Appendix 1. The resulting method was as follows: mucous cells were counted on 20 consecutive lamellae on both afferent and efferent sides (40 lamellae on each gill filament) on three filaments, that is 120 lamellae on each fish. The counts were performed blinded to sampling date and location with sections from all samplings mixed and counted by one person. Only cells with a distinct PAS-positive cytoplasm were counted using 63X magnification. Mucous cells in the interlamellar region were not included. The counts were performed in a proximal-to-distal direction from the basis of the filament. The filament situated approximately in the middle of the angle of the gill arch was counted first, followed by the next filament according to the sequence shown in Figure 1. The counted regions had to have an intact filament with a symmetrical distribution of at least 20 lamellae; otherwise, the next filament (in dorsal or ventral direction, respectively) was selected for counting (Figure 1). Oblique sections were re-orientated and re-processed for examination. Nine samples were not suited for counting due to sample irregularities and were discarded.

2.7 | Statistical investigations

The final data set ($n = 211$) is described in Table 1. The sum of all counted mucous cells from each fish ("total mucous cells") was used for all calculations if not stated otherwise. A linear regression model was built to investigate the variation in mucous cell counts across sites. The dependent variable was "total mucous cells," which was log-transformed to reach the assumption of normal distribution. The independent variables tested in the model were "fish weight," "fish length," "water transparency" and "fish farm." Variables were retained in the model if $p < .05$. The residuals from the final model were checked for normality and homoscedasticity. All data were first plotted in Microsoft Excel (Microsoft Corporation), and all statistical work was performed in StataSE 15 (Stata Corporation).

3 | RESULTS

3.1 | Weight and length

The median fish weight between the different farms varied between 74 and 246 g. The median weight of all fish was 124 g, and the mean was 144 g. The median length varied between 18.5 and 27 cm across

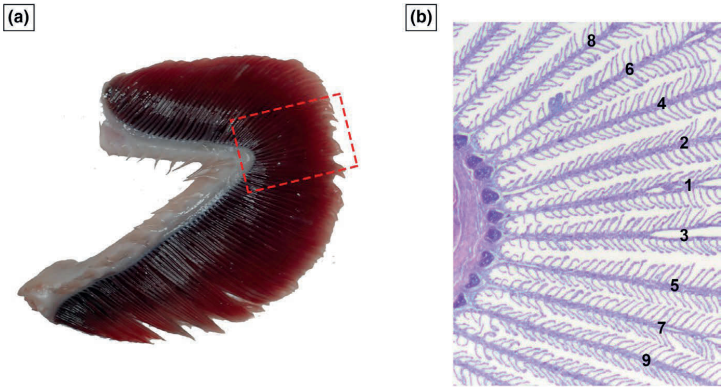

FIGURE 1 Mucous cell counting method. The starting point for the counting of each gill was determined in the middle of the angle of the gill arch (A—indicated by red box). The middle filament was termed 1 (B—indicated by 1). If the lamellae on this filament fulfilled the counting criteria (see Materials and Methods), mucous cells on this filament were counted. If not, counting of the next filament fulfilling the criteria was conducted. The selection of filament was performed in the order indicated in figure B until a total of three filaments had been counted. Mucous cells on 20 consecutive lamellae (see counting criteria in Materials and Methods) on both afferent and efferent sides were counted on each of the three filaments, that is 120 lamellae in total on each gill arch. Mucous cells in the interlamellar region were not counted

TABLE 1 Descriptive statistics for main variables, grouped by farm

Farm	Number of fish	Tank depth (cm)	Fish weight [g] *median (min–max)*	Fish length [cm] *median (min–max)*	Relative sight depth [%] *median (min–max)*	Mucous cell count *median (min–max)*
FT I	20	350	112 (56–218)	20 (17–24.8)	100	31 (21–73)
RAS I	38	400	74 (50–124)	18.5 (15.5–22)	58 (53–84)	44 (21–71)
RAS II	39	300	152 (52–284)	22 (16–29)	60 (54–63)	44 (16–257)
RAS III	39	400	246 (146–432)	27 (24–33)	100	89 (42–170)
RAS IV	39	410	95.5 (66.5–127)	20 (18–22)	100	68 (19–156)
RAS V	36	450	162 (111–218)	24 (21–27)	84 (57–84)	101 (35–216)
Total	**211**	**—**	**124 (50–432)**	**22 (15.5–33)**	**53–100**	**59 (16–257)**

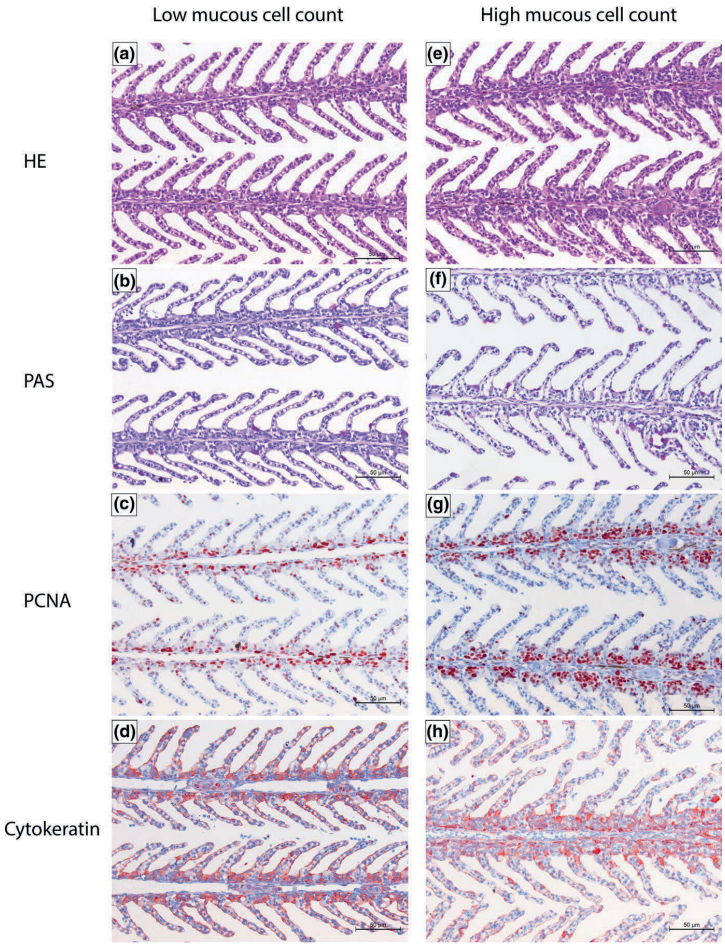

FIGURE 2 Histological investigations of gills with low (A-D) and high (E-H) mucous cell counts. A) HE stain showing gill filaments and lamellae of normal character. B) PAS stain showing sparse amounts of scattered PAS-positive cells on the lamellae. C) PCNA stain revealing proliferating cells at the base between the lamella (red colour). Scattered PCNA-positive cells are evident in the lamellae. D) Cytokeratin stain showing a dense epithelial network (red) in the interlamellar region. Cytokeratin reactivity is also evident in the respiratory epithelium of the lamellae. E) HE stain showing gills with a thickened and cell-rich filament. F) PAS stain showing multiple PAS-positive cells on the lamellae. A focal cluster of hyperplasia with many PAS-positive cells is evident in the lower right corner. G) PCNA stain revealing a proliferative response in the interlamellar region which appear thickened. H) Cytokeratin stain showing epithelial hyperplasia of basally located cells in the interlamellar epithelium. The stain appears less dense and organized than in image D. Pockets of non-epithelial cells are found within the epithelium

the six farms, and the median length of all fish was 22 cm (mean 22 cm). The median values of weight and length are described in Table 1.

3.2 | Water transparency

Water transparency varied between 54% and 100% sight depth in relation to the tank depth. The lowest and the highest records of sight depth in each farm are shown in Table 1. In three of the farms (FT I and RAS III-IV), the bottom of the tank was visible at both sampling points. The tank depth varied between 300 and 450 cm in the different farms (Table 1).

3.3 | Histopathological assessment

Histopathological changes were observed in only one fish with areas of subepithelial leucocyte infiltrates, epithelial cell hyperplasia and mucous cell hyperplasia.

3.4 | Immunohistochemistry—PCNA and cytokeratin

PCNA staining of gills with low mucous cell counts (Figure 2a and b) revealed proliferative cells at the base between lamellae, that is the interlamellar region of the filament (Figure 2c). Scattered positive cells were evident in the lamellae. Cytokeratin staining coincided with the PCNA stain, showing a dense red stain in the interlamellar region (Figure 2d). Additionally, the pavement cells of the lamellae were cytokeratin-positive. Gills with high mucous cell counts (Figure 2e and f) had a strong PCNA reaction in the interlamellar region, which also appeared thickened (Figure 2g). Cytokeratin staining of such gills was paler and more loosely organized than in gills with low mucous cell counts (Figure 2h). Infiltrates of other, non-epithelial cells were also evident in the interlamellar region.

3.5 | Mucous cell counts

The total number of mucous cells for each fish varied between 16 and 257, with a median of 59 (mean: 70.5). The median (min–max) values of mucous cell count from fish in each farm varied between 31 (21–73) as the lowest median and 101 (35–216) from the farm with the highest median value (Table 1 and Figure 3). The distribution of mucous cells in relation to fish weights is displayed in Figure 4. Further, the scatter plot in Figure 5 shows the relationship between mucous cell counts and fish length in each farm.

3.6 | Statistical analysis

The final regression model included "fish length" and "fish farm" as independent variables. "Fish length" and "fish weight" were highly correlated (0.96), and length was chosen because of higher explanatory power in the model. "Water transparency" was not statistically significant as a predictor of mucous cell count in the model. The final model had an adjusted R-square value of 0.44. If "fish farm" was removed, the adjusted R-square for the model was reduced to 0.23. Fitted values versus residuals indicated that the assumption of homoscedasticity was met, and the model residuals showed a normal distribution.

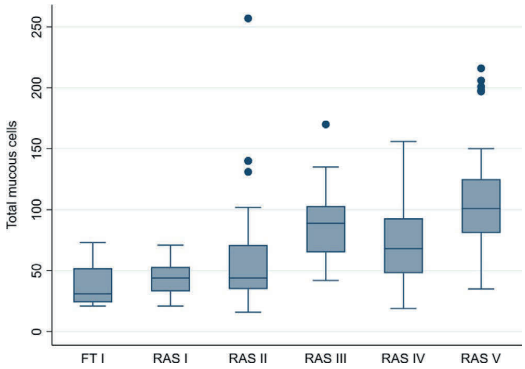

FIGURE 3 Distribution of total mucous cell count in each fish between the different farms [Colour figure can be viewed at wileyonlinelibrary.com]

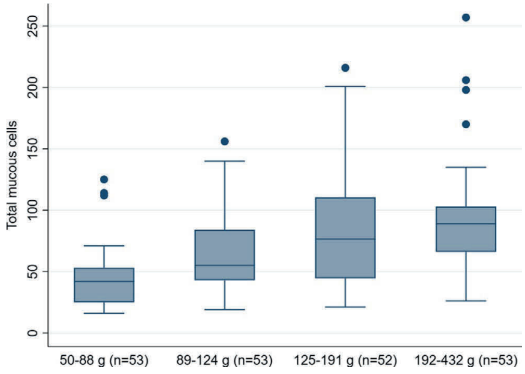

FIGURE 4 Total mucous cell count in relation to fish weight. The fish are split into equal groups by weight [Colour figure can be viewed at wileyonlinelibrary.com]

4 | DISCUSSION

To our knowledge, this is the first study describing the variation in mucous cell count from gills of clinically healthy salmon reared in commercial fish farms in Norway. Based on earlier publications, we have developed a method of counting mucous cells from salmon gill histology samples. The results indicate that variations in the number of mucous cells depend on farm-related factors, when fish length is

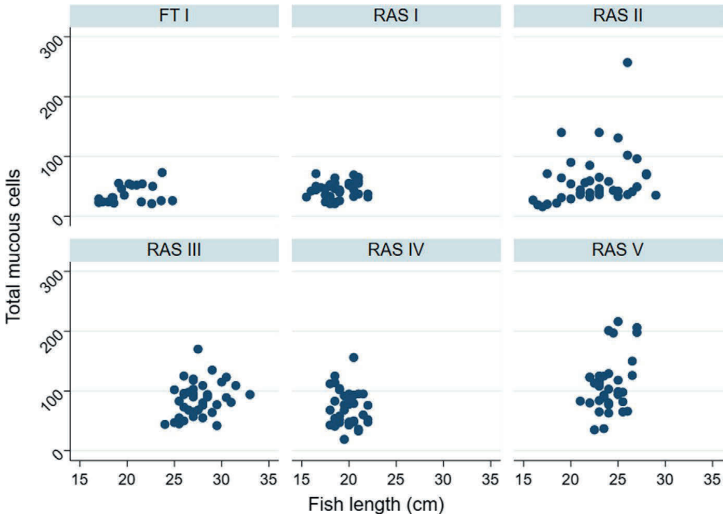

FIGURE 5 Scatter plot to show the distribution of total mucous cells counted in each fish by fish length [Colour figure can be viewed at wileyonlinelibrary.comwileyonlinelibrary.com]

accounted for by the regression model. This suggests that factors related to the fish farm will have an impact on the number of mucous cells in each fish. Immunohistochemical investigations indicated an early proliferative response in the gill epithelium correlating with increased mucous cell counts.

Mucous cells can be identified by several different staining methods and can be quantified using different approaches. Ferguson et al. (1992) acquired the number of mucous cells per a given gill area, while Speare et al. (1997) described a mucous cell index based on total counts of all lamellae on a filament. Others have taken into account the size of each mucous cell, in addition to the distribution, in a stereology-based method as described in Pittman et al. (2011) and Dang et al. (2019). Based on the work by Ferguson et al. (1992) and Speare et al. (1997), we have developed a modified method, where mucous cells are counted on the gill lamellae according to given criteria (see Appendix 1). To ensure accurate cell counts, mucous cells in the interlamellar region were not included. The high cell proliferation rate in this region made counting of single PAS-positive cells difficult; thus, only the lamellae were counted. The method proved suitable for our study, and the output variable (mucous cell count) was owed significant results in the regression model.

In this study, the number of mucous cells in the gill lamellae of clinically healthy fish was investigated. According to our counting method, filaments with less than 20 symmetrical pairs of lamellae were rejected and thus not counted. The method therefore excluded gills with common pathological changes such as lamellar hyperplasia. However, if there were focal pathological changes in the gills, but the other areas of the filaments fulfilled the counting criteria, the sample was counted and included in the study. In total, only one out of 211 gill samples showed pathological changes, making it difficult to conclude on how focal pathological changes affected the number of mucous cells.

Our results indicate fish size is associated with the number of mucous cells found in the gills. This is not unexpected in order to keep a constant ratio of mucous cells versus epithelial cells during growth. However, regarding median values of mucous cells from each farm, fish from RAS I and RAS II had a median of mucous cells close to the FT farm (FT I), and the median of RAS V was more than twice as high as RAS I and RAS II. At the same time, the weight and length of the fish in RAS II and RAS V were similar, indicating that some other factor than the size must explain the variation in mucous cell counts observed between farms. No gill diseases were reported in any of the fish groups included in the study. In the regression model, size of the fish (fish length) and fish farm as fixed effects together accounted for 44% of the variation in mucous cell counts. Meanwhile, the size alone only accounted for 23% of the variation explained by the model. This means that in these data, when the size of the fish is accounted for, factors within the fish farm had a substantial contribution to the proportion of explained variation in the number of mucous cells counted from each fish. Noteworthy, the lowest mean of mucous cells was found in the FT farm. One could speculate that favourable environmental conditions in the flow-through environment coincided with a low mucous cell count. However, the result should be interpreted with caution, as the material from the FT farm was limited to 20 fish from one sampling. Regarding the matter of individual or farm-related factors, fish from FT I and RAS IV were equivalent in size but fish from RAS IV on average had double the mucous cell counts compared to the FT farm. However, further studies are needed to tease out which farm-level factors contribute to the variation in mucous cell count, as well as to establish causal pathways on how, for example, management and the environment in the farms affect the mucous cell numbers.

The plastic responsiveness of mucous cells makes them important first-line defenders in the epithelial lining of the gills. Mucous cells are modified, highly polarized epithelial cells that produce and secrete

mucins at their apical surface. Hyperplastic and metaplastic mucous cell responses are commonly seen in several infectious gill diseases (Ferguson, 2006) but also with other stressors such as formalin treatment or high ammonia concentrations (Ferguson et al., 1992; Speare et al., 1997). It thus seems likely that other alterations in the environment, for example variable water parameters, may affect mucous cell dynamics and epithelial cell homeostasis. The cellular response in gills with both high and low mucous cell counts, respectively, was investigated using IHC targeting PCNA, a conserved marker for proliferation. Gills with low mucous cell counts revealed PCNA-positive cells mainly restricted to the interlamellar region, consistent with the location of the stem cell niche of the lamellae (Ferguson, 2006). Cytokeratin staining confirmed that these cells were mainly epithelial cells with a prominent and dense staining pattern. Gills scored with a high mucous cell count showed marked proliferation in a thickened interlamellar region. Cytokeratin staining revealed a paler staining pattern of more loosely arranged epithelial cells, allowing the presence of, for instance, leucocytes, reflecting an inflammatory reaction. Taken together with the high mucous cell count, this indicates an early organ response and in this case possibly towards environmental factors.

Water transparency measurements with Secchi disc proved difficult to perform in land-based facilities. Strong water currents and variation in light conditions caused inaccuracies during measuring across the tanks and thus represent a source of error in this variable. In three of the farms, the bottom of the tank was visible at both sampling occasions, reducing the variation of this parameter in the data set. However, the results obtained indicate no relationship between water transparency in the tanks and mucous cell counts in the gills. Given the uncertainty of the water transparency measurements, these results should be interpreted with caution. Future studies on the matter should include alternative methods to assess water quality, for example turbidity.

In conclusion, this cross-sectional study shows a variation in gill mucous cell counts in between six different commercial salmon farms, and more than 200 healthy salmon. This suggests that mucous cell counts can become a monitoring tool for gill health in the future.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

DP planned the study, sampled the material, carried out statistical analysis and wrote the manuscript. HB planned the study, carried out histological investigations and wrote the manuscript. AF planned the study, sampled the material, counted the mucous cells and commented on the manuscript. LAH planned the study, sampled the material, counted the mucous cells and commented on the manuscript. EOK planned the study, evaluated immunohistochemistry and commented on the manuscript. AN contributed to drafting of the manuscript, aided in statistical analyses and commented on the manuscript. MS planned the study, carried out statistical analysis, supervised the study and commented on the manuscript.

DATA AVAILABILITY STATEMENT

The data set generated in the study is not included but is available from the corresponding author on reasonable request.

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REFERENCES

Akvakultudriftsforskriften. (2008). *Forskrift om drift av akvakulturanlegg*. Retrieved from https://lovdata.no/forskrift/2008-06-17-822/§34

Awata, S., Tsuruta, T., Yada, T., & Iguchi, K. (2011). Effects of suspended sediment on cortisol levels in wild and cultured strains of ayu *Plecoglossus altivelis*. *Aquaculture*, *314*(1), 115–121. https://doi.org/10.1016/j.aquaculture.2011.01.024

Bancroft, J. D., & Gamble, M. (2008). *Theory and practice of histological techniques*. London, UKChurchill Livingstone.

Becke, C., Schumann, M., Steinhagen, D., Geist, J., & Brinker, A. (2018). Physiological consequences of chronic exposure of rainbow trout (*Oncorhynchus mykiss*) to suspended solid load in recirculating aquaculture systems. *Aquaculture*, *484*, 228–241. https://doi.org/10.1016/j.aquaculture.2017.11.030

Bjørgen, H., Koppang, E. O., Gunnes, G., Hordvik, I., Moldal, T., Kaldhusdal, M., & Dale, O. B. (2018). Ectopic epithelial cell clusters in salmonid intestine are associated with inflammation. *Journal of Fish Diseases*, *41*(7), 1031–1040. https://doi.org/10.1111/jfd.12780

Bruton, M. N. (1985). The effects of suspensoids on fish. *Hydrobiologia*, *125*(1), 221–241. https://doi.org/10.1007/BF00045937

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

Dalsgaard, J., Lund, I., Thorarinsdottir, R., Drengstig, A., Arvonen, K., & Pedersen, P. B. (2013). Farming different species in RAS in Nordic countries: Current status and future perspectives. *Aquacultural Engineering*, *53*, 2–13. https://doi.org/10.1016/j.aquaeng.2012.11.008

Dang, M., Pittman, K., Bach, L., Sonne, C., Hansson, S. V., Søndergaard, J., … Nowak, B. (2019). Mucous cell responses to contaminants and parasites in shorthorn sculpins (*Myoxocephalus scorpius*) from a former lead-zinc mine in West Greenland. *Science of the Total Environment*, *678*, 207–216. https://doi.org/10.1016/j.scitotenv.2019.04.412

Evans, D. H., Piermarini, P. M., & Choe, K. P. (2005). The multifunctional fish gill: Dominant site of gas exchange, osmoregulation,

acid-base regulation, and excretion of nitrogenous waste. *Physiological Reviews*, *85*(1), 97–177. https://doi.org/10.1152/physrev.00050.2003

Ferguson, H. W. (2006). *Systemic pathology of fish: A text and atlas of normal tissues in teleosts and their responses in disease*. London, UKScotian Press.

Ferguson, H. W., Morrison, D., Ostland, V. E., Lumsden, J., & Byrne, P. (1992). Responses of mucus-producing cells in gill disease of rainbow trout (*Oncorhynchus mykiss*). *Journal of Comparative Pathology*, *106*(3), 255–265. https://doi.org/10.1016/0021-9975(92)90054-X

Gjessing, M. C., Steinum, T., Olsen, A. B., Lie, K. I., Tavornpanich, S., Colquhoun, D. J., & Gjevre, A.-G. (2019). Histopathological investigation of complex gill disease in sea farmed Atlantic salmon. *PLoS One*, *14*(10), e0222926. https://doi.org/10.1371/journal.pone.0222926

Gomez, D., Sunyer, J. O., & Salinas, I. (2013). The mucosal immune system of fish: The evolution of tolerating commensals while fighting pathogens. *Fish & Shellfish Immunology*, *35*(6), 1729–1739. https://doi.org/10.1016/j.fsi.2013.09.032

Hjeltnes, B., Bæverfjord, G., Erikson, U., Mortensen, S., Rosten, T., & Østergård, P. (2012). *Risk assessment of recirculation systems in salmonid hatcheries* (09–808-Final). Retrieved from https://vkm.no/download/18.3a33d0ea16122420c393dc33/1516971511354/Risk%20Assessment%20of%20Recirculation%20Systems%20in%20Salmonid%20Hatcheries.pdf

Koppang, E. O., Kvellestad, A., & Fischer, U. (2015). Fish mucosal immunity: Gill. In B. H. Beck, & E. Peatman (Eds.), *Mucosal health in aquaculture* (pp. 94–120). London, UKAcademic Press.

Kristensen, T., Åtland, Å., Rosten, T., Urke, H. A., & Rosseland, B. O. (2009). Important influent-water quality parameters at freshwater production sites in two salmon producing countries. *Aquacultural Engineering*, *41*(2), 53–59. https://doi.org/10.1016/j.aquaeng.2009.06.009

Pittman, K., Sourd, P., Ravnøy, B., Espeland, Ø., Fiksdal, I. U., Oen, T., … Sweetman, J. (2011). Novel method for quantifying salmonid mucous cells. *Journal of Fish Diseases*, *34*(12), 931–936. https://doi.org/10.1111/j.1365-2761.2011.01308.x

Roberts, S. D., & Powell, M. D. (2003). Comparative ionic flux and gill mucous cell histochemistry: Effects of salinity and disease status in Atlantic salmon (*Salmo salar* L.). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *134*(3), 525–537. https://doi.org/10.1016/S1095-6433(02)00327-6

Rodger, H. D. (2007). Gill disorders: An emerging problem for farmed Atlantic salmon (*Salmo salar*) in the marine environment? *Fish Veterinary Journal*, *9*, 38–48.

Rogers, D. (1994). Airway goblet cells: Responsive and adaptable frontline defenders. *European Respiratory Journal*, *7*(9), 1690–1706. https://doi.org/10.1183/09031936.94.07091678

Speare, D. J., Arsenault, G., MacNair, N., & Powell, M. D. (1997). Branchial lesions associated with intermittent formalin bath treatment of Atlantic salmon, *Salmo salar* L., and rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Journal of Fish Diseases*, *20*(1), 27–33. https://doi.org/10.1046/j.1365-2761.1997.d01-103.x

Whitsett, J. A., & Alenghat, T. (2015). Respiratory epithelial cells orchestrate pulmonary innate immunity. *Nature Immunology*, *16*(1), 27–35. https://doi.org/10.1038/ni.3045

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APPENDIX 1

PRELIMINARY COUNTING METHOD

We specified the selection of filaments and lamellae by several criteria. To be suitable for counting, the criteria demanded normal morphological appearance of the 20–26 first (at the base of the filament) lamellae on both sides of the filament, including symmetry in length and width in lamellae. Thus, the basis for the mucous cell numbers in this paper was consistently obtained from proximal parts of the filaments. If the lamellae at the basis of the filament were irregular, the count could start more distal on the lamella (up to the 6th pair), given that the next 20 consecutive lamellae were acceptable for counting. Only lamellae fulfilling these criteria were counted; however, the selection of different filaments was random.

PRELIMINARY MUCOUS CELL COUNTS

To evaluate the counting method, 48 random slides were counted by two independent examiners. The mucous cell counts from each filament were summed, giving a total number of mucous cells for each fish. Examiner 1 (AF) had a median of 40.5 mucous cells (range 16–140) per fish, and examiner 2 (LAH) had a median of 44.5 mucous cells (range 13–150) per fish. The correlation between the examiners was 95.7%. The mean inter-examiner difference on each slide was 7.7 mucous cells (SE: 0.8). There was no apparent inter-relation between the inter-examiner difference and the number of mucous cells. Fourteen of the 48 slides had an inter-examiner difference of more than ten mucous cells, and among those, the mean inter-examiner difference was 14.9 (SE: 0.9).

Based on the preliminary counts, an additional criterion was added to specify the order of selection of filaments. The filament situated approximately in the middle of the angle of the gill arch was counted first, followed by the next filament according to the sequence shown in Figure 1. The counted regions had to have an intact filament with a symmetrical distribution of at least 20 lamellae; otherwise, the next filament (in dorsal or ventral direction, respectively) was selected for counting until three filaments were counted (Figure 1).

After re-evaluating the 14 slides with the additional criterion, four of the slides were rejected by both examiners. These slides were remade, resulting in three acceptable and one rejected slide. The rejected slide was discarded, leaving 13 slides in the group. The mean inter-examiner difference in this group decreased to 6.0 (SE: 0.8), and the mean inter-examiner difference among all slides in the evaluation ($n = 47$) decreased to 5.1 (SE: 0.4) mucous cells. The overall correlation between examiners improved to 98.1%. The 34 slides with an inter-examiner difference below ten mucous cells were determined to be adequate, and thus, these were not re-counted.

All remaining slides were counted by one examiner following the improved counting method, as described in Materials and Methods.

Paper IV

Bacterial community composition in gill mucus and corresponding environment in four commercial Atlantic salmon RAS

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Abstract

The use of recirculating aquaculture systems (RAS) in land-based fish farming has increased during the last years. Producing fish in those conditions requires knowledge about how this built environment affects the fish and how to effectively monitor fish health. The microbiome of fish is suggested to be associated with the immune system, with potential interactions to both the environment and the host. These mechanisms are poorly understood in general, and the aim of the study was to characterize microbiomes present in the gill mucous of healthy Atlantic salmon pre-smolt and the corresponding production environment in four commercial RAS facilities, and to investigate sources of variation in microbial communities. Gill mucus from 160 pre-smolts in four RAS facilities (RAS II-V) were sampled, together with environmental samples (tank water and biofilm) and subjected to 16S rRNA gene sequencing analyses. Quantification of 16S rRNA gene copy numbers in the extracted DNA from gill mucus indicated low bacterial abundance in general. A total of 67 gill samples were successfully sequenced, with substantial variation in bacterial profiles between the facilities. Fish from RAS II stood out, where all gill mucus samples were sequenced, and this correlated to the highest number of 16S rRNA gene copies. Interestingly, RAS II was the only farm practicing continuous production, indicating a higher bacterial abundance in gill mucus with this regime. Microbial communities in gills were dissimilar across the facilities (beta diversity), and the communities of gills and environment were also distinct different, where the environment showed higher alpha diversity (Shannon index). Assessed by Shannon diversity index as outcome in a regression model, sources of variation attributed most variation to the individual fish, suggesting gill microbiome structure was likely linked to the individual fish rather than the surrounding environment.

1. Introduction

Commercial production of Atlantic salmon (*Salmo salar*) is traditionally conducted in two phases. The first phase is carried out in land-based aquaculture systems (LBAS), typically comprising the development of juveniles from egg to smolt in freshwater or low salinity (0–3‰) brackish water, followed by the second phase for grow-out after transfer to open sea cages. LBAS are either flow-through systems (FTS) or recirculating aquaculture systems (RAS). Traditionally, FTS have been the dominating form of LBAS in Norway, utilizing water from a nearby river or lake, by redirecting the water to the farm. However, the amount and quality of water available at the source sets the premises for production, and limits the number of fish produced (Kristensen, Åtland, Rosten, Urke, & Rosseland, 2009). To overcome this challenge RAS technology was developed, which made it possible to reuse and recirculate the water within the farm. The effect was reduced water consumption, and the water source was no longer a limitation for an increased production (Dalsgaard et al., 2013). Water re-use and recirculation in RAS has the potential to contribute to improved sustainability and increased control of the water quality. Simultaneously, intensive water treatment and monitoring of an extended number of water quality parameters has become mandatory (Hjeltnes et al., 2012).

Advanced water treatment technology of RAS rapidly became the standard when building new on-land facilities for salmon production (Dalsgaard et al., 2013). Also, since growth in production at sea currently is restricted due to governmental regulation and environmental requirements, multiple land-based facilities producing salmon from egg to market-size are being built in Norway and other countries (Bjørndal & Tusvik, 2019). In these projects RAS-technology is vital to achieve the desired production within economic and environmental restrictions. Hence, it is crucial to understand more about how the RAS-environment affects the fish and how to effectively monitor fish health.

Fish farming is an intensive animal husbandry, and it is essential to provide a production environment with stable living conditions ensuring good health and welfare for the animals (Dahle et al., 2020; Drønen et al., 2021; Fossmark, Attramadal, Nordøy, Østerhus, & Vadstein, 2021). Consequently, monitoring the environment and fish health is important. Today there are several methods in use to monitor various water parameters (such as oxygen, carbon dioxide, pH, turbidity etc.) in the production environment (Kristensen et al., 2012; Kristensen et al., 2009). However, there are few methods available to monitor fish health (Bateman et al., 2021; Peeler & Taylor, 2011). Preferably the assessment of fish health should be performed to detect subclinical diseases, or alterations in the immune system. Such information would provide more precise knowledge for decision making at the farm in order to keep the fish healthy and avoid diseases and mortality.

Persson et al. (2020) described gill mucus cell count as a potential monitoring tool of fish health. Increase in mucus production is one of the initial responses of the immune system upon activation (Gomez, Sunyer, & Salinas, 2013; Rogers, 1994), and could therefore act as an early warning of deteriorating health status of the fish in production. The fish microbiome is also suggested to be a part of the immune system in mucosal surfaces, living in symbiosis with the host (Gomez et al., 2013; Llewellyn, Boutin, Hoseinifar, & Derome, 2014). This is a complex relationship, which typically supports the host suppressing intrusive agents but also could facilitate infection (González, Elena, & Prasad, 2021; Stevens, Bates, & King, 2021). These interactions between the host mucus microbiome and immune system are poorly understood, as well as how microbial ecosystems from the surrounding environment affect this interplay.

RAS represent built environments harbouring complex microbial ecosystems which differ considerably from natural environments. Bacteria are introduced through the intake-water, feed, fish itself and can vary in concentration and community composition over time (Blancheton, Attramadal, Michaud, d'Orbcastel, & Vadstein, 2013). The microbial community composition is distinct across the compartments of the RAS system and rearing water environment (Bakke et al., 2017; Bartelme, McLellan, & Newton, 2017) but in addition across different farms (Dahle et al., 2020; Fossmark et al., 2021; Minich et al., 2020). Several studies also describe the host-associated microbiome found on salmon skin, gills and digestive tract (Bozzi et al., 2021; Karlsen et al., 2017; Lokesh & Kiron, 2016; Minich et al., 2020; Minniti et al., 2017). However, there is scarce knowledge about the drivers and sources of variation of microbial diversity in salmon farming. Berggren et al. (2022) described high variability of microbial diversity between populations and individuals when investigating wild perch (*Perca fluviatilis*) (Berggren et al., 2022). Investigating sources of variation would be of importance also in salmon farming, towards the understanding of where in the production monitoring and intervention of the microbial ecosystem would benefit fish health the most.

The aim of the here described study was to characterize microbiomes present on salmon gills and in the corresponding production environment (water and biofilm) within four commercial salmon smolt RAS facilities in Norway. A secondary objective was to investigate sources of variation in the microbial diversity found on the gills and if the diversity correlates with the number of gill mucous cells.

2. Material and Methods

2.1 Material

Production system

The study was based on the sampling of fish gill mucus in addition to water (in tank and in RAS-unit) and biofilm (from biofilter and from tank wall) from four commercial RAS facilities producing Atlantic salmon smolts (Table 1). The facilities were located at the Western and Northern coasts of Norway and were named RAS II – V, corresponding to names given in Persson et al. (2020). RAS I was not sampled for sequencing analyses of gill microbiome and therefore excluded in this study. All farms had different designs of the production system and rearing conditions (Table 1) in the RAS-unit (RAS-unit defined as the tanks within the farm sharing biofilter and water environment). RAS III-V had an "all in - all out" protocol in the RAS-unit, meaning the fish was moved in and out of all tanks in the RAS-unit simultaneously. RAS II had a continuous production where fish were moved in and out of the RAS-unit independently of other tanks in the unit. The number of tanks in each studied RAS-unit varied from 4 to 12, the tank volume was between 318 to 783 m3 and total tank volume of the RAS unit in the different farms varied between 1584 to 4698 m3. Biomass capacity varied between 60 to 67 kg/m3. There were two different genetic breeds of salmon used at the different farms, where the two breeds were used in two farms each.

Table 1. Descriptive statistics of selected production parameters of the different hatcheries (RAS II-V) included in the study. Microbial diversity (Shannon index) at each sampling (in each tank) is also indicated. nd= no data

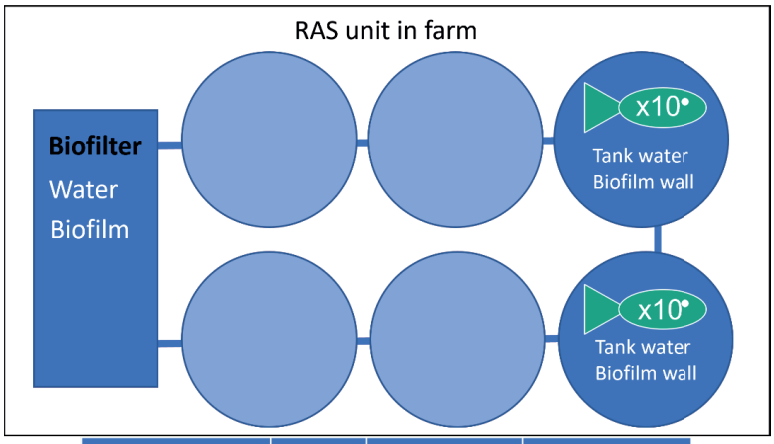
Farm	RAS II				RAS III				RAS IV				RAS V			
Number of tanks in RAS-unit	12				4				6				4			
Tank volume (m3)	318				396				783				900			
Biomass capacity in tank (kg/m3)	60				63				64				67			
Biomass capacity in RAS-unit (kg)	228 960				99 792				300 672				241 200			
Total tank volume in RAS-unit (m3)	3 816				1 584				4 698				3 600			
Genetic breed	A				A				B				B			
Sampling	Sampling 1		Sampling 2		Sampling 1		Sampling 2		Sampling 1		Sampling 2		Sampling 1		Sampling 2	
Number of fish in RAS-unit	982 927		1 215 578		401 232		401 213		1 555 976		1 555 212		816 439		816 003	
Avarage weight of fish in RAS-unit (g)	109		125		209		258		109		125		130		175	
Biomass in RAS-unit (kg)	107 513		152 312		83 857		103 620		170 145		194 837		106 528		142 890	
Proportion of biomass capacity in RAS-unit (%)	47		66		84		104		57		65		44		59	
Temperature (° C)	12.2		13.4		14.0		nd		8.6		8.0		14.1		14.0	
Salinity (‰)	0		0		15		15		2		2		4		4	
Tank	Tank 1	Tank 2	Tank 1	Tank 2	Tank 1	Tank 2	Tank 1	Tank 2	Tank 1	Tank 2	Tank 1	Tank 2	Tank 1	Tank 2	Tank 1	Tank 2
Number of fish in tank	103 121	80 758	103 084	80 620	100 119	100 119	100 113	100 114	306 579	339 126	306 504	339 007	204 440	203 816	204 306	203 678
Mortality between sampling (‰)	-	-	0,36	1,71	-	-	0,06	0,05	-	-	0,24	0,35	-	-	0,66	0,68
Weight of sampled fish in tank, mean (g) [sd]	77 [16]	157 [17]	140 [42]	213 [31]	228 [43]	225 [39]	266 [43]	286 [79]	95 [9]	87 [11]	104 [15]	95 [9]	145 [25]	142 [28]	196 [31]	171 [22]
Biomass in tank (kg/m3)	32	39	45	55	51	53	63	67	34	35	42	41	30	28	39	38
Proportion of maximum biomass in tank (%)	53	66	75	92	81	85	101	106	54	54	65	64	45	42	58	56
Shannon index mean [sd]	3.5 [0.7]	2.9 [0.5]	3.0 [0.4]	3.6 [0.4]	2.4 [0.1]	3.2 [nd]	1.9 [nd]	nd	1.9 [nd]	nd	3.6 [0.1]	4.1 [0.9]	3.3 [0.8]	4.0 [1.2]	2.7 [1.3]	2.7 [1.0]
Shannon index number of samples sequenced	10	10	10	10	2	1	1	0	1	0	3	2	5	4	3	5

Sampling

Fish

Gill mucus and histology samples from a total of 160 clinically healthy fish were collected from the four RAS from November 2018 to January 2019. Two samplings of 20 fish from two different tanks

(10 fish from each tank) were conducted at each RAS with 14 days between the samplings (Figure 1). The samplings were performed in the time-period between vaccination and sea transfer.



Samples	2 tanks	2 time points	Total 4 farms
Fish (10 per tank)	20	40	**160**
Total environment	6	12	**48**

Figure 1. Illustration of the sampling performed in the study. In one RAS-unit in each farm, 10 fish from 2 tanks (at two timepoints) were sampled. From the environment, water (in the tanks and the RAS loop) and biofilm (from tank wall and biofilter) were sampled at the same timepoints as the fish. The table show the total amount of samples from the fish and environment (water and biofilm).

The fish were gently netted out from the tanks and euthanized by an overdose of sedation (Finquel© vet, Scan Aqua), in line with regulations of the Norwegian Directorate of Fisheries (Akvakulturdriftsforskriften, 2008). Weight and length were recorded. The sampling procedure of gill mucus was based on the skin sampling technique described by Minniti et al. (2017). Gill mucus was sampled with a swab (Copan Diagnostics, USA) gently swabbing across the surface of all gill arches on the right side. The swab was placed back into the container (no transport media used), stored on ice during transport and frozen (-20 °C) at the lab until further preparation. The second gill arch from the left side was stored in formalin for further histology preparations at the lab.

RAS system environment (Water and biofilm)

Water and biofilm samples were collected at the same time-points as the fish (Figure 1). Water samples were from two rearing tanks and water in the RAS loop in each farm. Biofilm samples were taken from the tank wall (in each tank) and the biofilter.

Sampling of water and biofilm was performed as described in Dahle et al. (2020). Briefly, water samples were collected by filtering 150-200 mL water through a 0.22 µm Sterivex filter (Millipore, USA) with 60 ml Omnifix® syringes. Biofilm samples were taken by swabbing (Copan Diagnostics,

USA) the tank walls of the two rearing tanks and inside the fixed bed biofilter. The samples were stored in freezers (-20 °C at farms, -80 °C at lab) until further analyses were performed.

2.3 Methods

DNA-extraction

DNA extraction from gill mucus samples was performed with QIAamp BiOstic Bacteremia DNA Kit (Qiagen) according to the protocol with minor alterations to facilitate sample material deriving from swabs (step 1-2 was omitted). Quantity and quality of extracted DNA was assessed employing Qubit 3.0 and dsDNA HS (High Sensitivity) Assay Kit (Thermo Fisher Scientific), and Nanodrop 1000 (Thermo Fisher Scientific) measuring 260/280 and 260/230 nm wavelength ratios, respectively.

From water and biofilm, DNA was extracted with ZymoBIOMICS™ DNA Miniprep kit (Zymo Research, USA), as described by the manufacturers. The Genomic DNA Clean & Concentrator™-10 kit (Zymo Research, Irvine, California) was used to purify the DNA.

DNA-sequencing and microbiome data analyses

Gill samples which passed the quality threshold required by the sequencing protocol (n=110) and environment (water and biofilm, n=47) was sent to the Centre for Biotechnology (CeBiTec), Bielefeld University (Germany) for 16S rDNA amplicon library preparation and sequencing. Library preparation was conducted after standard Illumina instructions. The variable regions 3 and 4 (v3 + v4) of the 16S rRNA gene was amplified by two PCR rounds using the 2xHiFi HotStart ReadyMix (Kapa Biosystems, USA). To cover the domains of Bacteria and Archaea, the primers 341F (CCTACGGGNGGCWGCAG) and 805R (GACTACHVGGGTATCTAATCC) were used for the first PCR round (Klindworth et al., 2013). Obtained amplicons were indexed, pooled and subsequently sequenced on an Illumina MiSeq platform (paired end sequencing; 2x300 bp).

The microbiome data analyses were performed in QIIME2, pipeline version 2020.2.0 (Bolyen et al., 2019). Raw sequences were quality check (trim 20 bp, truncate forward 290 bp and reverse 270 bp) denoised, merged, checked for chimeric sequences and amplicon sequence variants (ASV) classifications using dada2 was performed (Callahan et al., 2016). A phylogenetic tree was created, and taxonomy was assigned using Silva database release v.138 (Parks et al., 2018; Quast et al., 2013). Chloroplast and mitochondria associated ASV were removed, as well as ASV annotated to Bacteria and Proteobacteria without further annotation and “unassigned” ASV at kingdom level. In addition, Thermaceae was removed due to possible contamination from the PCR-process (Corless et al., 2000). ASV with less than 5 reads were filtered out, and the ASV table rarefied to 4000 reads. Thus, 43 gill samples were removed due to the normalization. Leaving 67 gill samples for further analyses.

All the analyses related to microbial ecology were performed in R 4.1.0. Alpha microbial diversity was calculated using “phyloseq” R package with the Shannon diversity index as the metric of choice. The comparison of the alpha diversity between the environmental and fish microbiomes was done by using Mann-Whitney-Wilcoxon test.

Beta microbial diversity was calculated with the “vegan” R package. This was done by non-metric multidimensional scaling (NMDS) on the Bray-Curtis dissimilarity matrix followed by visualization on a biplot and a permutational multivariate analysis of variance (PERMANOVA, 9999 permutations) using the “adonis” function of the “vegan” package.

To detect differentially abundant ASVs, the ’corncob’ package was run on the ASV-table by fitting a beta-binomial regression model to microbial data with the “farm” variable as a covariate (FDR cut-off=0.01 (type 1 error rate), Wald hypothesis test).

Quantification of bacterial abundances employing digital PCR

Analysis of bacterial abundance in fish mucus samples was conducted by absolute quantification of 16S rRNA gene copy numbers in corresponding DNA extracts using the Naica Crystal digital PCR (dPCR) System (Stilla Technologies). The detailed dPCR workflow was performed as described elsewhere (Netzer, Ribičić, Aas, Cavé, & Dhawan, 2021). In short, for each sample, a 25 µl reaction mixtures was prepared (1x concentrated PerFecTa Multiplex qPCR ToughMix (Quanta Biosciences), 1 µM fluorescein, 1 µM of primers, 250 nM of corresponding TaqMan probe, and an appropriate amount of DNA template) and loaded on a Sapphire chip. Sample partitioning and PCR was performed in Sapphire chips in the Naica Geode using the following program: 1. 95°C for 5 min, 2. 95°C for 30 sec and 57°C for 30 sec, 45 cycles. In addition, also the abundance of host DNA was assessed by quantification of 18S rRNA gene copies in corresponding DNA extracts with dPCR as described above applying following PCR conditions: 1. 95°C for 5 min, 2. 95°C for 30 sec and 60°C for 30 sec, 50 cycles. Oligonucleotide sequences for corresponding primers and TaqMan probes are provided in Table 2. Data analysis was performed employing Crystal Miner software V2.3.5 (Stilla Technologies). For all assays, reactions with no template (NTC) were performed to control for DNA contaminations.

Table 2. Oligonucleotide sequences of primers and TaqMan-probes bacterial 16S rRNA genes and eukaryotic 18S rRNA gene.

Primer and probe name	Sequence (5'-3')	Target gene	Reference
1055f	ATGGCTGTCGTCAGCT	16S	Harms et al., (2003)
1392r	ACGGGCGGTGTGTAC	16S	Harms et al., (2003)
16STaq1115-CY5	CY5-CAACGAGCGCAACCC-BHQ2	16S	Harms et al., (2003)
18S-O-F	CCCCGTAATTGGAATGAGTACACTTT	18S	Olsvik et al., (2005)
18S-O-R	ACGCTATTGGAGCTGGAATTACC	18S	Olsvik et al., (2005)
18S-O-Taq-FAM	FAM-CACCAGACTTGCCTCC	18S	Olsvik et al., (2005)

Histology and mucous cell count

Gill samples on formalin were processed and stained with hematoxylin and eosin (HE) for histological investigations and periodic acid-Schiff (PAS) for the detection of mucins according to standard procedures (Bancroft & Gamble, 2008). Mucous cell count was performed according to the method described in Persson et al. (2020). Briefly, mucous cells were counted on 40 lamellae on three gill filaments on each gill histology sample (20 consecutive lamellae on both sides of each filament). Mucous cells in the interlamellar region were not included. The counted regions had to have an intact filament of at least 20 lamellae, otherwise the next filament was selected for counting. The filament situated in the middle of the gill arch was the starting point for counting. Out of the 160 fish sampled, 153 gill histology samples were used to count mucous cells. The remaining seven were not suitable for counting due to sample irregularities.

Sources of variation

To investigate sources of variation in gill microbiome diversity a two-level regression model was built with microbial diversity (expressed as Shannon index) as outcome and sampling event as random effect (sampling event: grouping variable combining farm, tank and sampling time point) and the individual salmon (i.e., gill sample) as the bottom level. The outcome reached the assumption of normal distribution. Only samples from farms RAS II and V, which had successfully sequenced samples from all sampling points and tanks, was included in a subset used for the analysis (RAS II: n=40, RAS V: n= 17) (Table 1). Only production parameters affecting the fish level were tested as fixed effects (fish weight and mucous cell count). Variables were assessed as significant if $p<0.05$. Production parameters at other levels (farm, tank or sampling point) were excluded in the model-building due to low statistical power at these levels, leading to a high correlation between parameters and the grouping variable (sampling event). To estimate the variation attributed to each level in the model, variance component proportion (VCP) was calculated (Dohoo, Martin, & Stryhn, 2009).

Data management and statistical work

Information about the production facilities and production data was collected from the farms, plotted in Excel (Microsoft corp) and transferred to Stata (Stata/SE 15.0, Stata corp) where one complete metadatafile was assembled and some descriptive statistics and multilevel modelling was performed. The metadata was transferred into R-studio (R-core team) where all further analyses in conjunction with the microbiome data were performed.

3. Results

3.1 Production system and management

Measurements from each sampling day and tank are displayed in Table 1. All fish appeared healthy when examined at sampling. Weight at the time of sampling varied from 77 g (sd: 16 g) in the tank with the smallest fish at sampling 1, to 286 g (sd: 79 g) in the tank with the largest fish at sampling 2 within the dataset. The farm with the largest difference in weight between the tanks was RAS II. Mortality in the tanks between sampling varied between 0.04 to 1.7 ‰. The biomass increased between sampling in each tank, and at the second sampling the biomass in each tank varied from 38 to 67 kg/m3. Expressed as a proportion of the maximum allowed biomass in tank, this varied between 56 to 106 % at the sampling 2. Water temperature was around 14 °C in RAS III and RAS V, 12-13 °C in RAS II and 8-9 °C in RAS IV at the sampling time points. Salinity varied between 0 and 4 ‰ in RAS II, IV and V. In RAS III it was 15 ‰.

3.2 Total DNA and 16s rRNA ratio

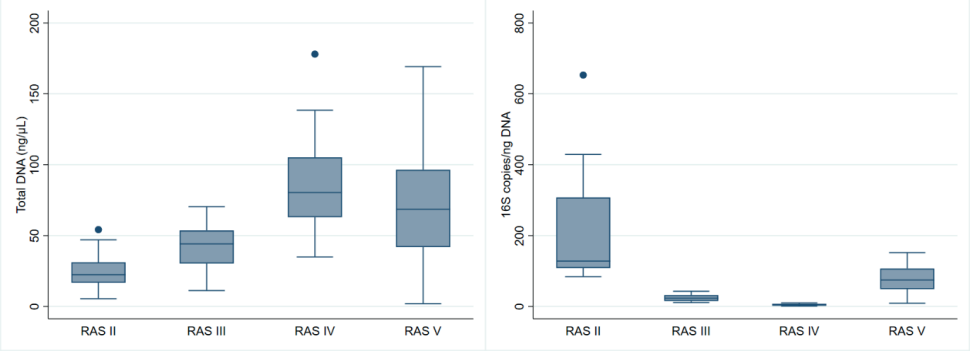


Figure 2. Boxplot of Total DNA in the graph to the left and 16s rRNA copies (per ng DNA) on the right

The concentration of total DNA extracted from gill mucus samples and corresponding 16S rRNA gene copies per ng total DNA in the subset is shown in Figure 2. The mean DNA concentration varied from 25 ng/ul in RAS II to 85 ng/ul in farm IV, with an overall mean of 55 ng/ul. There was a substantial

variation in 16S rRNA gene copies per ng DNA between the farms, ranging from 5 in RAS IV to 228 in RAS II, with an overall mean of 83. Indicating low abundance of bacteria in the gill mucus.

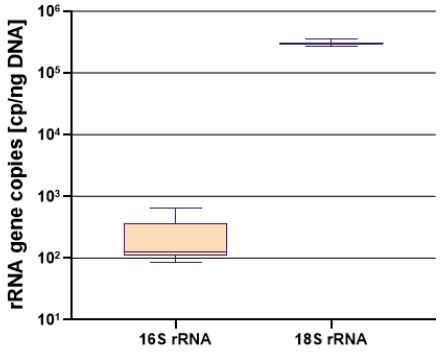


Figure 3. Abundance of 16S and 18S rRNA gene copies in total DNA extracted from gill mucus samples derived from RAS II facility.

To demonstrate that the vast majority of total DNA extracted from gill mucus was host DNA, 18S rRNA gene copies were quantified in three gill mucus samples derived from fish in RAS II and compared to 16S rRNA gene copies detected in samples from the same facility (Figure 3). Even in samples from RAS II, where the highest abundance of prokaryotic 16S rRNA gene copies was found, 99.7 % of rRNA molecules were attributed to the host's DNA.

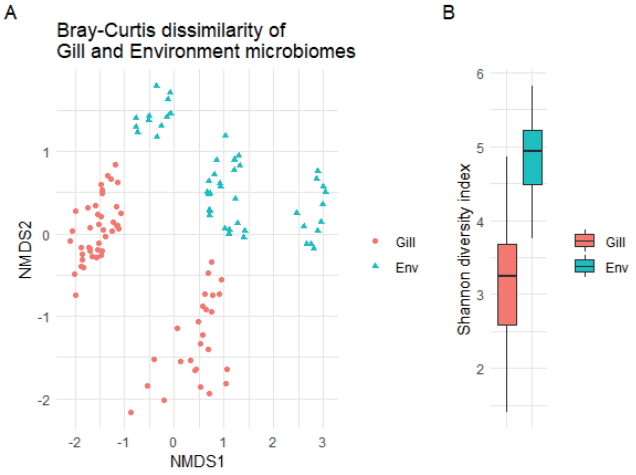


Figure 4. Microbial diversity of fish gills and RAS. Panel A: Beta diversity; non-metric multidimensional scaling (NMDS) of Bray-Curtis dissimilarities derived from the microbiomes of the fish gill and the RAS specimens across the whole dataset colored by the source of the samples (blue - RAS, red - fish gills). Panel B: Alpha diversity; Shannon diversity index is shown as a distribution of the Shannon diversity indices of the fish gill microbiomes (red box) and the RAS samples (blue box) in comparison. The boxes of the plot indicate the interquartile range (IQR) with the black horizontal line indicating the median values and whiskers indicating the lower and upper quartiles of the distribution). The results of the test comparing the two distributions are given in the text.

3.3 Microbial ecology of RAS and fish

A total of 67 gill samples and 47 environmental (env) samples were sequenced (RAS II: 40 gill and 12 env, RAS III: 4 gill and 12 env, RAS IV: 6 gill and 12 env. RAS V: 17 gill and 11 env). A total of 9476 unique ASV detected (5837 in samples from gill and 5672 in env), and assessed by rarefaction curves, samples had sufficient sequencing depth.

The microbial communities in the environment and gill mucus samples revealed distinct compositions as estimated by PERMANOVA on the Bray-Curtis dissimilarities as a proxy for beta microbial diversity ($F=20.8$, $p=0.0001$, 9999 permutations, Figure 4A). Subsequently, corresponding alpha microbial diversity (Wilcoxon $W = 102$, p-value $< 2.2e-16$) was different with on average higher Shannon indices found in the environmental microbiomes (4,89 [SD=0.52]) than gill mucus microbiomes (3.21 [SD=0.78]) (Figure 4B).

The microbial communities in the environment were distinct across the studied farms when assessed in terms of beta microbial diversity. The microbiomes of RAS V and RAS IV appeared more compositionally similar than that metric of RAS III and RAS II (PERMANOVA $F=14.5$, $R2 = 0.25$ against $F= 537.8$, $R2= 0.63$, respectively).

Relative abundance of the ten most frequent bacteria phylum detected in the gill and environment at each sampling time point (in each farm) is shown in Figure 5A-B. The pattern indicates distinct variation in the expression of different microbial taxa in the gill between the farms and also (to a lower extent) between the fish. The environmental samples appear more diverse and consistent between the farms (compared to the gills). However, there are still differences evident between farms and time of sampling. Eight of the phyla are overlapping between gill and environment (given the filtration of the ten most frequent phyla in each of gill and environment), leaving Chlamydiae and Nitrospirae exclusive to environment and Deinococcus-Thermus and Firmicutes in the gills.

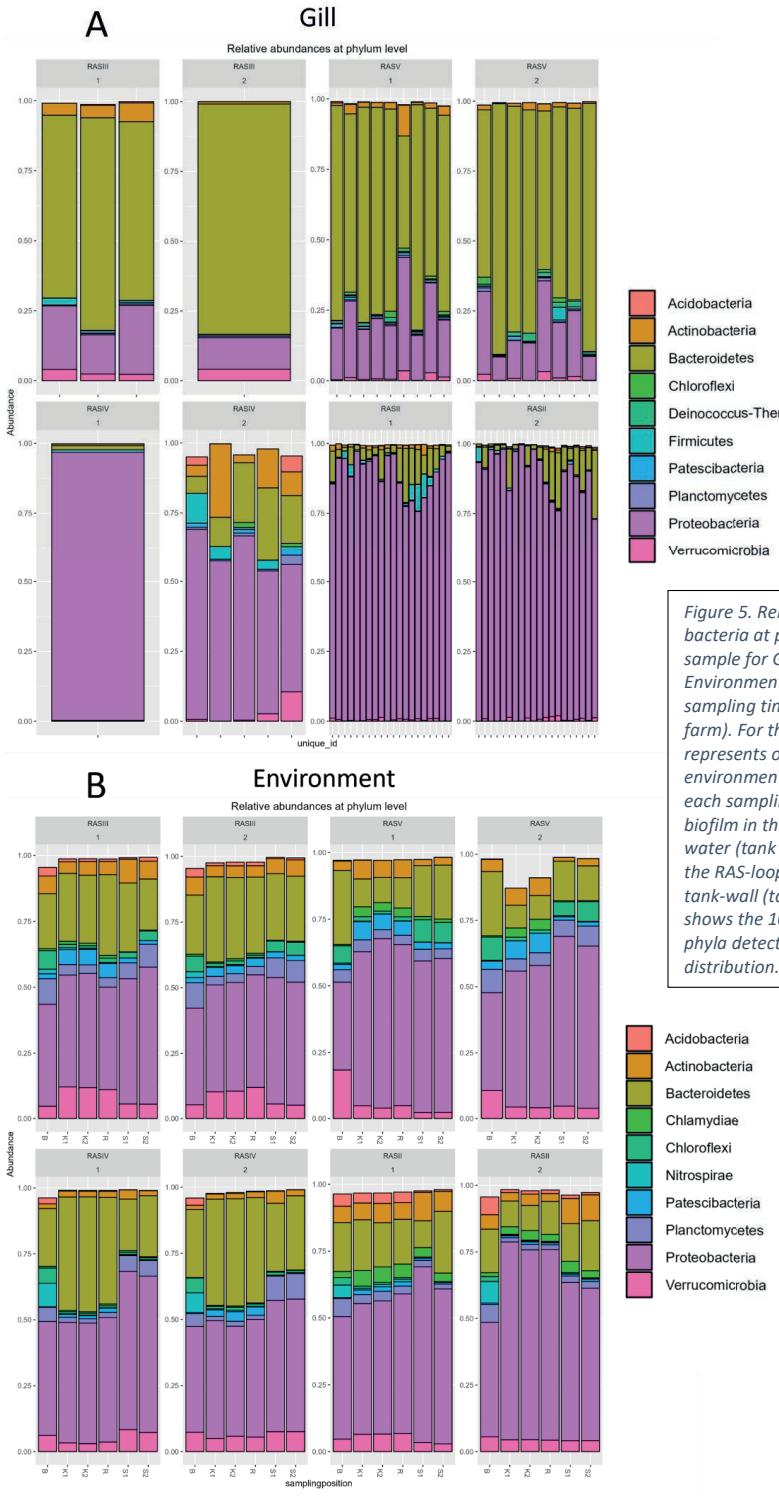


Figure 5. Relative abundance of bacteria at phylum level in each sample for Gill (A) and Environment (B). Grouped by sampling timepoint (in each farm). For the gill each bar represents one fish, and the environment have one bar for each sampling position (B: biofilm in the RAS-unit, K: tank-water (tank 1-2), R: water from the RAS-loop, S: biofilm from tank-wall (tank 1-2)). The figure shows the 10 most abundant phyla detected and their distribution.

Investigating the presence of taxa known to be involved in the nitrification process in the biofilter (ammonia oxidising and nitrite oxidising bacteria), this is visualised qualitative (Figure 6A-B) by identified families (Nitrosococcaceae, Nitrosomonadaceae, Nitrosopumilaceae and Nitrospiraceae) in gills and environment for RAS II and V at each sampling timepoint. The dominating families are Nitrospriaceae and Nitrosomonadaceae across farm and time, but with apparent differences in expression between both individual fish, sampling time points and environmental sample position.

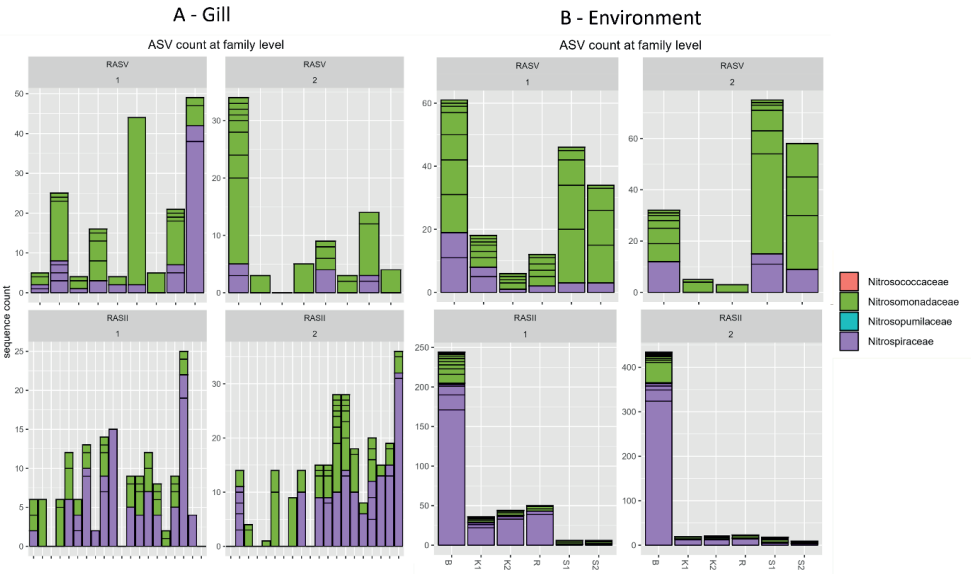


Figure 6. Qualitative visualization of taxa (number of ASV) known to be involved in the nitrification process in both gill (A) and environment (B). Grouped by farm (RAS II and V) and sampling timepoint (1 or 2). Each bar represents one sample, for the gill each fish, and for the environment the sampling position (B: biofilm in the RAS-unit, K: tank-water (tank 1-2), R: water from the RAS-loop, S: biofilm from tank-wall (tank 1-2)). Note the different scale for each sub-graph.

Gill mucus microbiomes

The microbial communities in the fish gill mucus were dissimilar across the farms (Figure 7). However, the gill mucus microbiota of RAS II stood apart in terms microbial compositions when RAS III-V were considered as one group and compared to RAS II (PERMANOVA $F=53$, $R2=0.45$, $p<0.0001$ at 9999 permutations).

The gill microbiomes of fish in RAS II were characterized by higher relative abundances of *Psychrobacter* and *Shewanella* genera ASVs compared to the other farms, as shown in Figure 8.

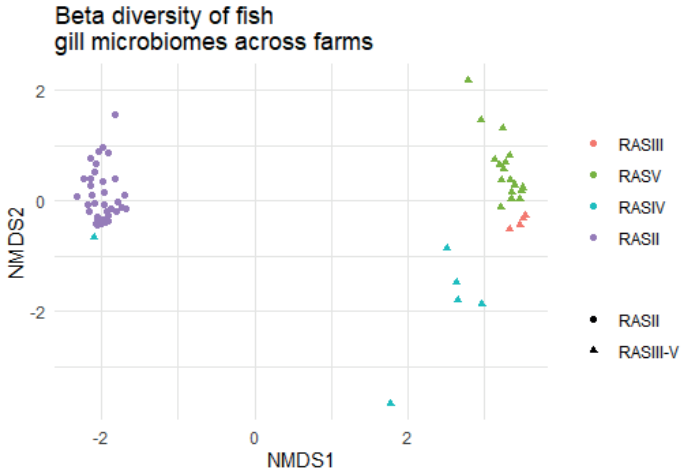


Figure 7. Non-metric multidimensional scaling (NMDS) of Bray-Curtis dissimilarities derived from the fish gill microbiomes across the farms. The samples are colored according to the farm affiliation (lilac-RASII, red-RASIII, cyan-RASIV, and green-RASV) and shaped as belonging to either RASII (circles) or the group comprised of the rest farms (triangles)

3.4 Histology and mucous cells

From the fish with successful sequencing results (n=67), mucous cell count was performed on 64. The median mucous cell count (min-max, n) was in RAS: II 44 (16-257, 39), RAS III: 101.5 (64-120, 4), RAS IV: 73.5 (35-89, 6) and RAS V: 94 (80-216, 15). Detailed results of the mucous cell count from all fish at the farms are presented in Persson et al. (2020).

3.5 Sources of variation in Shannon diversity

None of the fixed effects were significant in the model. Hence, the null model was used to explore sources of variation. Random effect of sampling event had an estimate in the model of 0.06 (SD: 0,08) and estimate of the residuals was 0.5 (sd: 0.1). VCP for sampling event was 11% (95% confidence interval: 1-60%). Leaving 89% of the variation attributed to the individual fish in this two-levelled model. The limited effect of sampling event on microbial Shannon diversity is visible when displayed graphically to investigate where the variation resides (Figure 9).

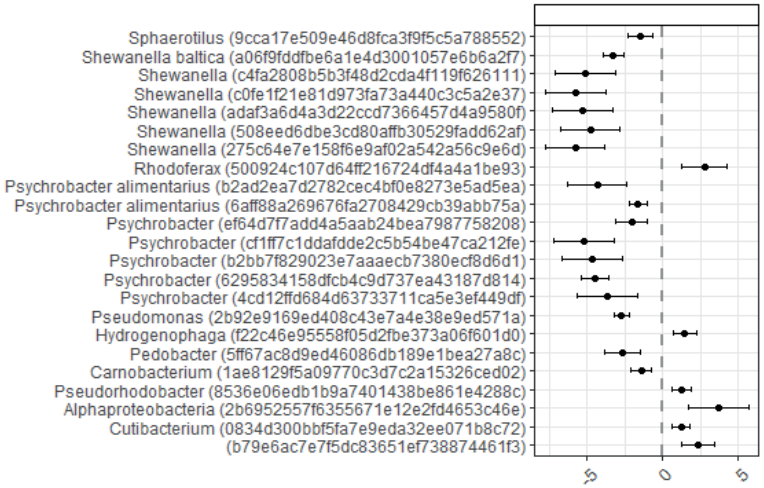


Figure 8. The figure shows the summary of the differential abundance testing using "Corncob" r package, on Y axis the ASVs that are differentially abundant are listed with their genus names and the hash values (in brackets) to differentiate among ASVs with identical genus names. The estimates and their confidence intervals derived from the model are represented on the graph. Negative values on the left of 0 (negative log change) should indicate lower relative abundance of a given amplicon in the RASIII-RASV group microbiomes compared to those of RASII. Positive values on the right of 0 (negative log change) should indicate higher relative abundance of a given amplicon in the RASIII-RASV group microbiomes compared to those of RASII microbiomes

4. Discussion

The major objective in this study was to identify potential relationships between the microbial communities in different commercial RAS for Atlantic salmon smolt and corresponding fish gill mucus, and histological mucous cell analyses. The alpha microbial diversity appeared to be different between the environment and the fish gill mucus. Further, gill mucus microbiome beta diversity divided the samples in two compositionally distinct groups. The gill microbiomes of fish from RAS II were compositionally distinct, when compared to the other farms.

In this study several of the gill samples did not pass the quality control threshold sufficient for sequencing. Unlike water and biofilm samples, where all samples passed the quality control. Whether this was due to an actual biological difference in amount of detectable DNA-material or due to the different protocols for DNA-extraction used (or other undetected challenges in the analytic process) was not possible to conclude upon. Therefore, to take this uncertainty into account, comparison between the two niches sampled (gills and environment) have been limited in this study and the gill samples have been the main focus.

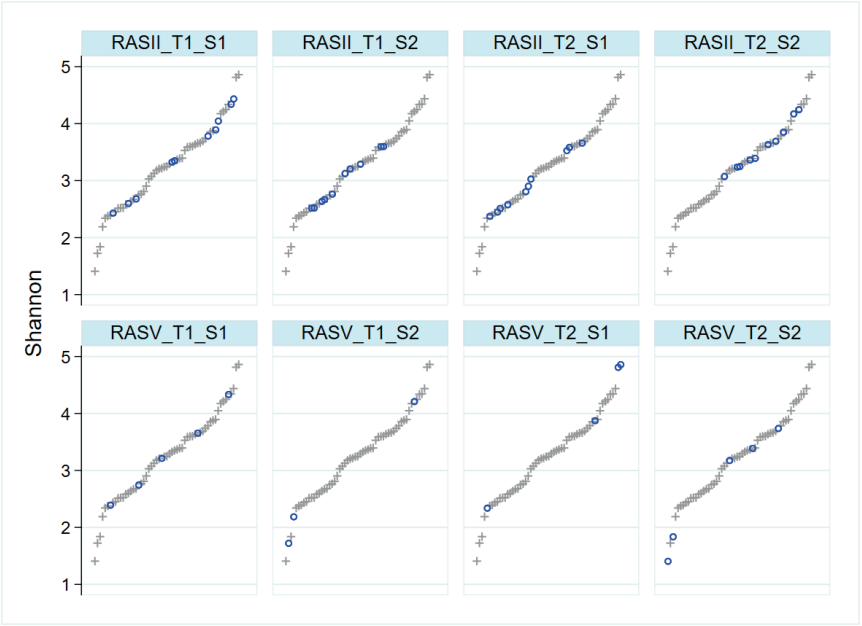


Figure 9. *Graphical overview of variation in microbial diversity (Shannon index) in gill samples from RAS II and RAS V. Grey cross-marks in each plot show all samples in the subset (n=57, samples from RAS II and RAS V). Blue circles highlight the samples representing each group (as labelled in each plot) in relation to the overall variation between samples in the dataset. Each plot is labelled according to farm [RAS II or V], tank [T1 or T2 in each farm] and sampling [S1 or S2 from each tank]). The samples are sorted by value of Shannon index, from lowest to highest.*

Assessed by Shannon index as outcome in the regression model, sources of variation analyses attributed most variation to the individual fish. The combined contribution of farm, tank and sampling time point ("sampling event") as random effect was modest. This suggests that the gill microbiome structure was likely linked to the individual fish rather than to the surrounding environment and management factors for example. There was no evident association between mucous cell count and microbiome diversity

All fish housed in the farms, before and between samplings, were reported healthy and the production parameters were within normal values according to the facilities. Mortality was low when assessed as total mortality during the 14-day sampling interval. There are few publications about mortality in salmon hatcheries with RAS. Recent publications from the Norwegian veterinary institute (Gåsnes et al., 2021; Tørud, Bang Jensen, Gåsnes, Grønbech, & Gismervik, 2019) have investigated mortality records from all hatcheries in Norway (FT and RAS), and they reported a mean mortality per month of 0.5 to 1 % for similar weight classes as those in our study. Compared to those mortality numbers, the mortality in our study was very low.

Assessed from the beta diversity of the gills, RAS II grouped separately from the other farms. RAS II also had the highest number of 16S rRNA gene sequences detected. Explored further, gill mucus

from fish in RAS II harboured more bacterial species affiliated to *Psychrobacter* and *Shewanella* genera compared to fish from RAS III-V. Investigating relative abundance, gill mucus from RAS II had a larger proportion of bacterial taxa from the phyla *Protebacteria* compared to RAS III-V. There was no correlation detected between specific production parameters and microbial diversity (estimated by Shannon index). However, one farm managing practice only performed in RAS II was continuous production, meaning that fish was moved in and out of tanks in the RAS-unit independently, keeping biomass of fish stable. The other three farms had an all-in-all-out approach where all fish was moved in and out of the unit simultaneously in all tanks, meaning the biomass of fish will vary considerably over time. The all-in all-out approach will reduce the risk of breaching biosecurity and spreading of diseases when different fish-groups are kept separate in the production and allows fallowing all tanks in the unit simultaneously (Ervik et al., 2020; Larsen et al., 2020). With continuous production, the fish biomass in the RAS-unit would be kept more constant over time. Potentially providing more stable production conditions for the fish in a way that cannot be observed in the other RAS facilities with a cleaning between each production cycle in the unit. Another finding separating RAS II from RAS V was the expression of different taxa involved in nitrifying processes in the RAS, here Nitrospiraceae was the most detected family in RAS II (both in the environment and in the gills). In RAS V Nitrosomonadaceae dominated. In terms of production strategies, no studies have so far indicated which of the two management practices is the most beneficial for the fish. Data from an increased number of farms would be necessary. However, an indication of production performance is the increase in weight of the fish between the two samplings in this study, and this was higher in RAS II compared to the other facilities.

As stated earlier, several of the gill samples had insufficient quality for sequencing. This was addressed by the estimation of the proportion of bacterial DNA to the total DNA in a selection of samples from each farm. Despite the presence of DNA in samples from all farms, only samples from RAS II showed a substantial amount of 16S rRNA copies. This proportion was low in RAS III and RAS IV samples suggesting a presence of low levels of bacteria in the specimens. These results also correlate to the number of successfully sequenced samples from the farms, where RAS II had all the samples sequenced, RAS V having approximately 40% of the samples successfully sequenced. While only 10% and 15% of the samples collected at RAS III and RASIV, respectively, passed the quality threshold required by the sequencing protocol. However, even though the number of successfully sequenced samples were not evenly spread between the farms, undetected errors with the analyses or at sampling cannot be ruled out as the cause of the large number of samples that did not pass quality control at sequencing.

To further explore the relation between host and bacterial DNA, a few samples from RAS II were subjected to quantification of 18S rRNA copies, specifically designed to quantify salmon DNA (Olsvik et al., 2005). RAS II was the farm with the most detected 16S rRNA copies and the lowest total DNA concentration and even here approximately 98% of the rRNA molecules were attributed to the host. In total, this information indicates generally low levels of bacteria found in gill mucus of fish from the RAS facilities in this study. However, with distinct variations between farms.

Sources of variation in microbial diversity was investigated on a subset of gill samples (RAS II and RAS V), with Shannon diversity index as outcome. The production hierarchy is important to consider and structure the data accordingly when building the regression model (Persson, Nødtvedt, Aunsmo, & Stormoen, 2021). The fish constituted the lowest level, fish was further reared in tanks within each farm. In addition, the timepoint for sampling would also have a potential influence and accounted for. Hence, the preferred model would have considered all this individually and nested in each other. However, since there was a restricted number of samples available for the analyses this was not possible. Instead, the grouping variable "sampling event" was constructed and combined the structure of farm, tank and sampling timepoint into one variable creating eight groups of fish. Visual assessment of the relation between gill microbial diversity and sampling event indicated a variation in diversity independent of the constructed group-variable. Results from the regression model emphasized this indication, where a major part of the variation was attributed to the individual sample. The VCP of the combined farm-tank-time level was only 11%. However, the confidence interval was wide, reflecting the low number of groups included. Emphasizing the need for studies to be performed across several farms to further investigate this finding.

The statistical model indicates that the individual fish is the major contributor to the variation in the microbial diversity in salmon gills. Meaning that the host microbiome modulates independently of the surrounding environment. Similar results were found in a study on two geographically different population of perch in southern Sweden, where most variation in microbial composition was explained by the individual host (Berggren et al., 2022). Specific individual microbial composition was also described in a study by Karlsen et al. (2017) investigating skin mucus microbiome of salmon with ulcers in a marine farm over time (Karlsen et al., 2017). Minich et al. (2020) found that microbial diversity (measured by UniFrac) was driven by body site (gill, skin and gut), hatchery and then tank, when investigating salmon from three different hatcheries. They concluded that the association between tank and fish microbiome was strong, however, they found no significant differences of gill microbiome between the farms (Minich et al., 2020). A recent study, following fish in the last part of the freshwater phase (at one farm) and the transition to seawater, found that gill and skin mucus-associated microbial communities were temporally dynamic in a RAS and with

distinct differences in microbial composition between fresh- and sea water (Lorgen-Ritchie et al., 2022). Other studies conclude that the environment alters the host associated microbiome extensively, however, at the same time also describing an individual variation between the fish in each group (Uren Webster, Consuegra, Hitchings, & Garcia de Leaniz, 2018; Uren Webster et al., 2020). Minniti et al. (2017) investigated skin microbiota before and after netting of salmon in an experimental setting. The authors found that there was individual variation between fish but concluded that there was no effect of the rearing water on the microbiota. However, they found that the microbiome becomes more uniform (assessed by relative abundance) within the fish-group after netting (Minniti et al., 2017). Comparison of studies of microbiome compositions are challenging, emphasized by the documented difficulties to reproduce sequencing results from the bacterial 16S rRNA gene and alteration in which primers are used to target the gene (Bharti & Grimm, 2019; Klemetsen, Willassen, & Karlsen, 2019; Sinha et al., 2017).

One aim of this study was to investigate whether the mucous cell count of the gills and the gill microbial diversity estimated by the Shannon diversity index were associated. The results indicated no relation between these two measurements. This is in line with a previous study, even though they used UniFrac as the diversity measurement of the microbiome (Minich et al., 2020). The same study found a significant correlation between mucous cells and skin microbiota, also between gill microbiota and number of goblet cells in the gut. However, the causal relationship between these variables was not discussed further (Minich et al., 2020).

This cross-sectional study did not find any associations between the gill microbial diversity and production parameters. The living conditions for bacteria in a RAS are determined by multiple factors, such as feed, management routines, water parameters and selection pressure in the biofilter (Blancheton et al., 2013). In this study, RAS III had a salinity of 15 ‰ at both sampling time points, different from the other farms with 0-4 ‰. Seawater transfer have shown to re-shape the microbiota of the external mucosa (Lokesh & Kiron, 2016; Lorgen-Ritchie et al., 2022), indicating the importance of this environmental difference between the farms. However, this was not the only difference between RAS III and the other farms in terms of production parameters. For instance, RAS III also had the highest biomass in the tank (measured as proportion of biomass capacity of the RAS-unit), coinciding with that the fish was transferred to seawater the day after the last sampling time point (data not shown). Therefore, conclusion to whether a specific production parameter (e.g., salinity, temperature, weight of the fish etc) affected the microbial composition was not possible since there were only four farms studied. Further, the timeframe for sampling could have been optimized, as sampling timepoint of fish varied between post vaccination to just before sea transfer. The latter indicate that this fish had undergone smoltification. Some of this could have been

accounted for by registration of morphological traits separating parr from smolt at sampling (Folmar & Dickhoff, 1980). However, this was considered having limited impact on the result, given the above discussion of production parameters and limited number of farms.

The study indicates variation in gill microbiome diversity to be attributed to the host itself, only modestly affected by the production surroundings. This study has limitations with unbalanced numbers of successfully sequenced samples from the farms. However, this unbalance correlates with the variation in bacterial DNA of mucus samples between the farms, further indicating relatively low levels of bacterial DNA found in gill mucus in general. To investigate the interplay between production parameters, fish health and microbial expression further, longitudinal studies (as performed by Lorgen-Ritchie et al. (2022)) are needed to find how the production parameters affect the microbial ecology of the salmon within the production environment across farms. Microbiota is undoubtedly important for farmed fish, however, the importance of the interaction between gill microbiota and the host needs to be studied further. Moreover, few studies discuss the causality of observed changes in microbiomes of fish. If the immune system modulates the host-associated microbiome, or if the microbiome alters the immune system of the host is not known. Such knowledge would be of importance if monitoring of microbiome within salmon production should become an effective tool in fish health management.

5. References

Akvakulturdriftsforskriften. (2008). Forskrift om drift av akvakulturanlegg. Retrieved from https://lovdata.no/forskrift/2008-06-17-822/

Bakke, I., Åm, A. L., Kolarevic, J., Ytrestøyl, T., Vadstein, O., Attramadal, K. J. K., & Terjesen, B. F. (2017). Microbial community dynamics in semi-commercial RAS for production of Atlantic salmon post-smolts at different salinities. *Aquacultural Engineering, 78*, 42-49. doi:10.1016/j.aquaeng.2016.10.002

Bancroft, J. D., & Gamble, M. (2008). *Theory and Practice of Histological Techniques*. London: Churchill Livingstone.

Bartelme, R. P., McLellan, S. L., & Newton, R. J. (2017). Freshwater Recirculating Aquaculture System Operations Drive Biofilter Bacterial Community Shifts around a Stable Nitrifying Consortium of Ammonia-Oxidizing Archaea and Comammox Nitrospira. *Frontiers in Microbiology, 8*. doi:10.3389/fmicb.2017.00101

Bateman, A. W., Schulze, A. D., Kaukinen, K. H., Tabata, A., Mordecai, G., Flynn, K., . . . Miller, K. M. (2021). Descriptive multi-agent epidemiology via molecular screening on Atlantic salmon farms in the northeast Pacific Ocean. *Sci Rep, 11*(1), 3466. doi:10.1038/s41598-020-78978-9

Berggren, H., Tibblin, P., Yıldırım, Y., Broman, E., Larsson, P., Lundin, D., & Forsman, A. (2022). Fish Skin Microbiomes Are Highly Variable Among Individuals and Populations but Not Within Individuals. *Frontiers in Microbiology, 12*. doi:10.3389/fmicb.2021.767770

Bharti, R., & Grimm, D. G. (2019). Current challenges and best-practice protocols for microbiome analysis. *Briefings in Bioinformatics, 22*(1), 178-193. doi:10.1093/bib/bbz155

Bjørndal, T., & Tusvik, A. (2019). Economic analysis of land based farming of salmon. *Aquaculture Economics & Management, 23*(4), 449-475. doi:10.1080/13657305.2019.1654558

Blancheton, J. P., Attramadal, K. J. K., Michaud, L., d'Orbcastel, E. R., & Vadstein, O. (2013). Insight into bacterial population in aquaculture systems and its implication. *Aquacultural Engineering, 53*, 30-39. doi:10.1016/j.aquaeng.2012.11.009

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., . . . Caporaso, J. G. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology, 37*(8), 852-857. doi:10.1038/s41587-019-0209-9

Bozzi, D., Rasmussen, J. A., Carøe, C., Sveier, H., Nordøy, K., Gilbert, M. T. P., & Limborg, M. T. (2021). Salmon gut microbiota correlates with disease infection status: potential for monitoring health in farmed animals. *Animal Microbiome, 3*(1), 30. doi:10.1186/s42523-021-00096-2

491 Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J., & Holmes, S. P. (2016).
492 DADA2: High-resolution sample inference from Illumina amplicon data. *Nat Methods, 13*(7),
493 581-583. doi:10.1038/nmeth.3869
494 Corless, C. E., Guiver, M., Borrow, R., Edwards-Jones, V., Kaczmarski, E. B., & Fox, A. J. (2000).
495 Contamination and sensitivity issues with a real-time universal 16S rRNA PCR. *Journal of*
496 *clinical microbiology, 38*(5), 1747-1752. doi:10.1128/jcm.38.5.1747-1752.2000
497 Dahle, S. W., Bakke, I., Birkeland, M., Nordøy, K., Dalum, A. S., & Attramadal, K. J. K. (2020).
498 Production of lumpfish (Cyclopterus lumpus L.) in RAS with distinct water treatments: Effects
499 on fish survival, growth, gill health and microbial communities in rearing water and biofilm.
500 *Aquaculture, 522*, 735097. doi:10.1016/j.aquaculture.2020.735097
501 Dalsgaard, J., Lund, I., Thorarinsdottir, R., Drengstig, A., Arvonen, K., & Pedersen, P. B. (2013).
502 Farming different species in RAS in Nordic countries: Current status and future perspectives.
503 *Aquacultural Engineering, 53*, 2-13. doi:doi.org/10.1016/j.aquaeng.2012.11.008
504 Dohoo, I. R., Martin, S. W., & Stryhn, H. (2009). *Veterinary epidemiologic research*. Charlotte, P.E.I.:
505 VER, Inc.
506 Drønen, K., Roalkvam, I., Dahle, H., Nilsen, H., Olsen, A. B., & Wergeland, H. (2021). Microbial
507 colonization and community stability in a marine post-smolt RAS inoculated with a
508 commercial starter culture. *Aquaculture Reports, 20*, 100745.
509 doi:10.1016/j.aqrep.2021.100745
510 Ervik, L.-C., Larsen, J. S., Klakegg, B., Sandberg, M. G., Johansen, E., & Holmøy, R. (2020).
511 *Smittesikring og biosikkerhet i norsk lakseproduksjon, delrapport 1 (in Norwegian: Infection*
512 *controll and biosecurity in Norwegian salmon production, report 1)*. Retrieved from BDO and
513 Åkerblå: https://www.fhf.no/prosjekter/prosjektbasen/901522/
514 Folmar, L. C., & Dickhoff, W. W. (1980). The parr—Smolt transformation (smoltification) and
515 seawater adaptation in salmonids: A review of selected literature. *Aquaculture, 21*(1), 1-37.
516 doi:10.1016/0044-8486(80)90123-4
517 Fossmark, R. O., Attramadal, K. J. K., Nordøy, K., Østerhus, S. W., & Vadstein, O. (2021). A
518 comparison of two seawater adaptation strategies for Atlantic salmon post-smolt (Salmo
519 salar) grown in recirculating aquaculture systems (RAS): Nitrification, water and gut
520 microbiota, and performance of fish. *Aquaculture, 532*, 735973.
521 doi:10.1016/j.aquaculture.2020.735973
522 Gomez, D., Sunyer, J. O., & Salinas, I. (2013). The mucosal immune system of fish: The evolution of
523 tolerating commensals while fighting pathogens. *Fish & Shellfish Immunology, 35*(6), 1729-
524 1739. doi:10.1016/j.fsi.2013.09.032

González, R., Elena, S. F., & Prasad, V. R. (2021). The Interplay between the Host Microbiome and Pathogenic Viral Infections. *mBio, 12*(6), e02496-02421. doi:10.1128/mBio.02496-21

Gåsnes, S. K., Oliveira, V. H. S., Gismervik, K., Ahimbisibwe, A., Tørud, B., & Jensen, B. B. (2021). Mortality patterns during the freshwater production phase of salmonids in Norway. *Journal of Fish Diseases, 44*(12), 2083-2096. doi:10.1111/jfd.13522

Harms, G., Layton, A. C., Dionisi, H. M., Gregory, I. R., Garrett, V. M., Hawkins, S. A., . . . Sayler, G. S. (2003). Real-Time PCR Quantification of Nitrifying Bacteria in a Municipal Wastewater Treatment Plant. *Environmental Science & Technology, 37*(2), 343-351. doi:10.1021/es0257164

Hjeltnes, B., Bæverfjord, G., Erikson, U., Mortensen, S., Rosten, T., & Østergård, P. (2012). *Risk assessment of recirculation systems in salmonid hatcheries* (09-808-Final). Retrieved from https://vkm.no/download/18.3a33d0ea16122420c393dc33/1516971511354/Risk%20Assessment%20of%20Recirculation%20Systems%20in%20Salmonid%20Hatcheries.pdf

Karlsen, C., Ottem, K. F., Brevik Ø, J., Davey, M., Sørum, H., & Winther-Larsen, H. C. (2017). The environmental and host-associated bacterial microbiota of Arctic seawater-farmed Atlantic salmon with ulcerative disorders. *Journal of Fish Diseases, 40*(11), 1645-1663. doi:10.1111/jfd.12632

Klemetsen, T., Willassen, N. P., & Karlsen, C. R. (2019). Full-length 16S rRNA gene classification of Atlantic salmon bacteria and effects of using different 16S variable regions on community structure analysis. *MicrobiologyOpen, 8*(10), e898-e898. doi:10.1002/mbo3.898

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., & Glöckner, F. O. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Research, 41*(1), e1-e1. doi:10.1093/nar/gks808

Kristensen, T., Haugen, T. O., Rosten, T., Fjellheim, A., Åtland, Å., & Rosseland, B. O. (2012). Effects of production intensity and production strategies in commercial Atlantic salmon smolt (Salmo salar L.) production on subsequent performance in the early sea stage. *Fish Physiology and Biochemistry, 38*(1), 273-282. doi:10.1007/s10695-011-9566-0

Kristensen, T., Åtland, Å., Rosten, T., Urke, H. A., & Rosseland, B. O. (2009). Important influent-water quality parameters at freshwater production sites in two salmon producing countries. *Aquacultural Engineering, 41*(2), 53-59. doi:10.1016/j.aquaeng.2009.06.009

Larsen, J. S., Ervik, L.-C., Klakegg, B., Sandberg, M. G., Johansen, E., & Holmøy, R. (2020). *Smittesikring og biosikkerhet i norsk lakseproduksjon, sluttrapport (in Norwegian: Infection*

controll and biosecurity in Norwegian salmon production, final report). Retrieved from BDO and Åkerblå: https://www.fhf.no/prosjekter/prosjektbasen/901522/

Llewellyn, M. S., Boutin, S., Hoseinifar, S. H., & Derome, N. (2014). Teleost microbiomes: the state of the art in their characterization, manipulation and importance in aquaculture and fisheries. *Frontiers in Microbiology, 5*(207). doi:10.3389/fmicb.2014.00207

Lokesh, J., & Kiron, V. (2016). Transition from freshwater to seawater reshapes the skin-associated microbiota of Atlantic salmon. *Scientific Reports, 6*, 19707. doi:10.1038/srep19707

Lorgen-Ritchie, M., Clarkson, M., Chalmers, L., Taylor, J. F., Migaud, H., & Martin, S. A. M. (2022). Temporal changes in skin and gill microbiomes of Atlantic salmon in a recirculating aquaculture system – Why do they matter? *Aquaculture, 558*, 738352. doi:10.1016/j.aquaculture.2022.738352

Minich, J. J., Poore, G. D., Jantawongsri, K., Johnston, C., Bowie, K., Bowman, J., . . . Allen, E. E. (2020). Microbial Ecology of Atlantic Salmon (Salmo salar) Hatcheries: Impacts of the Built Environment on Fish Mucosal Microbiota. *Applied and Environmental Microbiology, 86*(12). doi:10.1128/aem.00411-20

Minniti, G., Hagen, L. H., Porcellato, D., Jørgensen, S. M., Pope, P. B., & Vaaje-Kolstad, G. (2017). The Skin-Mucus Microbial Community of Farmed Atlantic Salmon (Salmo salar). *Frontiers in Microbiology, 8*(2043). doi:10.3389/fmicb.2017.02043

Netzer, R., Ribičić, D., Aas, M., Cavé, L., & Dhawan, T. (2021). Absolute quantification of priority bacteria in aquaculture using digital PCR. *Journal of Microbiological Methods, 183*, 106171. doi:10.1016/j.mimet.2021.106171

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

Parks, D. H., Chuvochina, M., Waite, D. W., Rinke, C., Skarshewski, A., Chaumeil, P.-A., & Hugenholtz, P. (2018). A standardized bacterial taxonomy based on genome phylogeny substantially revises the tree of life. *Nature Biotechnology, 36*(10), 996-1004. doi:10.1038/nbt.4229

Peeler, E. J., & Taylor, N. G. H. (2011). The application of epidemiology in aquatic animal health - opportunities and challenges. *Veterinary Research, 42*(1), 94. doi:10.1186/1297-9716-42-94

Persson, D., Bjørgen, H., Figenschou, A., Hillestad, L.-A., Koppang, E. O., Nødtvedt, A., & Stormoen, M. (2020). Variations in mucous cell numbers in gills of Atlantic salmon (Salmo salar) presmolt in commercial freshwater farms in Norway. *Journal of Fish Diseases, 44*, 25-32. doi:10.1111/jfd.13263

Persson, D., Nødtvedt, A., Aunsmo, A., & Stormoen, M. (2021). Analysing mortality patterns in salmon farming using daily cage registrations. *Journal of Fish Diseases, 00*, 1-13. doi:10.1111/jfd.13560

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., . . . Glöckner, F. O. (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Research, 41*(Database issue), D590-596. doi:10.1093/nar/gks1219

Rogers, D. (1994). Airway goblet cells: responsive and adaptable front-line defenders. *European Respiratory Journal, 7*(9), 1690-1706. doi:10.1183/09031936.94.07091678

Sinha, R., Abu-Ali, G., Vogtmann, E., Fodor, A. A., Ren, B., Amir, A., . . . The Microbiome Quality Control Project, C. (2017). Assessment of variation in microbial community amplicon sequencing by the Microbiome Quality Control (MBQC) project consortium. *Nature Biotechnology, 35*(11), 1077-1086. doi:10.1038/nbt.3981

Stevens, E. J., Bates, K. A., & King, K. C. (2021). Host microbiota can facilitate pathogen infection. *PLOS Pathogens, 17*(5), e1009514. doi:10.1371/journal.ppat.1009514

Tørud, B., Bang Jensen, B., Gåsnes, S., Grønbech, S., & Gismervik, K. (2019). *Dyrevelferd i settefiskproduksjonen (In norwegian: Animal welfare in the hatcheries)*. Retrieved from Norwegian veterinary institute: https://www.vetinst.no/rapporter-og-publikasjoner/rapporter/2019/dyrevelferd-i-settefiskproduksjonen-smafiskvel

Uren Webster, T. M., Consuegra, S., Hitchings, M., & Garcia de Leaniz, C. (2018). Interpopulation Variation in the Atlantic Salmon Microbiome Reflects Environmental and Genetic Diversity. *Applied and Environmental Microbiology, 84*(16). doi:10.1128/aem.00691-18

Uren Webster, T.M., Rodriguez-Barreto, D., Castaldo, G., Gough, P., Consuegra, S., & Garcia de Leaniz, C. (2020). Environmental plasticity and colonisation history in the Atlantic salmon microbiome: A translocation experiment. *Molecular ecology, 29*(5), 886-898. doi:10.1111/mec.15369

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