Norwegian University of Life Sciences
Faculty of Veterinary Medicine
Department of Paraclinical Sciences

Philosophiae Doctor (PhD)
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Development, optimization and field testing of a filtration method for detection of salmonid alphavirus in seawater

Utvikling, optimering og felttesting av en filtreringsmetode for påvisning av salmonid alphavirus i sjøvann

Lisa-Victoria Bernhardt

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To my dear family

"Bringing nature into the classroom
can kindle a fascination and passion
for the diversity of life on earth
and can motivate a sense of responsibility
to safeguard it."

- David Attenborough (1926-)

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Acknowledgements

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I will be forever grateful for everything my dear family and friends did for me and for always being there for me day and night, both close and at a distance, through thick and thin ♥

Lastly, I would like to end this acknowledgements with a quote in my mother tongue (Swedish):

»Den som känner andra, är lärd.
Den som känner sig själv, är vis.
Den som besegrar andra, har muskelkrafter.
Den som besegrar sig själv, är stark.
Den som är nöjd med sitt, är rik.
Den som inte förlorat sin medelväg, hen bibehåller sig.»
(Lao Tzu)

Abbreviations

bp = Basepairs

CMS = Cardiomyopathy syndrome

ddPCR = Digital droplet polymerase chain reaction

DNA = Deoxyribo-nucleic-acid

eDNA = Environmental DNA

eRNA = Environmental RNA

HSMI = Heart and skeletal muscle inflammation

H&E = Haematoxylin and eosin

ILAB = The Industrial and Aquatic Laboratory

IFAT = Indirect Fluorescent Antibody

IHC = Immunohistochemistry

i.p. = Intraperitoneal

IPC = Internal process control

IPN = Infectious pancreatic necrosis

LOD = Limit of detection

LOQ = Limit of quantification

MDS+ = 1MDS electropositive filter

MF- = Electronegative nitrocellulose MF hydrophilic membrane filter

NVI = Norwegian Veterinary Institute

PCR = Polymerase chain reaction

PD = Pancreas disease

RNA = Ribonucleic acid

RT-qPCR = Reverse-transcription quantitative real-time polymerase chain reaction

RT-PCR = Reverse-transcription polymerase chain reaction

SAV = Salmonid alphavirus

SDV = Sleeping disease virus

Site = A cluster of net-pens that are sharing a restricted geographic area in water for aquaculture

SPDV = Salmon pancreas disease virus

+ssRNA = Positive-sense single-stranded RNA

ssRNA = Single-stranded RNA

T_H = High viral dose tank

T_L = Low viral dose tank

Summary

The commercial aquaculture is continuously growing with the demand for fish and seafood products. In Norway, Atlantic salmon (*Salmo salar* L.) constitute 94 % of the total aquaculture production, making it the most important species in Norwegian aquaculture. With the high demand of fish and fish products globally, the farming of salmonids continues to occur intensively. This result in fish being under constant threat of being infected by pathogens and suffer from different diseases. Pancreas disease (PD), caused by *salmonid alphavirus* (SAV), is a systemic disease and is considered among the most serious virus diseases in sea farmed salmonids with a negative impact on fish welfare. It is also one of the most economically important fish diseases in aquaculture in Europe. This disease was first reported in 1976 in Scotland and in the 1980s in Norway, where it became a notifiable disease (list 3) in 2007.

In 2017, the national surveillance program for PD was intensified in Norway, introducing a PD zone for SAV2 and SAV3 (i.e. Western- and Mid-Norway), and two surveillance zones north and south/south-east of the PD zone. This was done as a way to reduce the consequences of the disease, as well as to prevent further spread of SAV within the defined zones. This surveillance program relies on a time-consuming and resource-demanding approach, involving monthly sampling of at least 20 fish from all SAV-negative marine operative farm sites with salmonid fish, and analyzing heart tissue from each fish by RT-qPCR analysis.

For years, significant progress has been made in developing filtration methods for concentrating low amounts of pathogens in water, and surveillance programs for different types of harmful pathogens have already been established based on a filtration technique. Hence, the focus of this study was to develop and optimize a filtration method for the detection of SAV in seawater, making the selective and invasive traditional testing of fish redundant. This study was divided into three steps; initially tested *in vitro*, followed by evaluation in a challenge model, and assessed under natural field conditions.

The *in vitro* study was performed in order to test five combinations of two different electrocharged filters and four different buffer solutions for concentration and detection of SAV3 in seawater by spiking SAV3 into 1 L of artificial and natural seawater. The SAV3 was quantified by using RT-qPCR and RT-ddPCR in order to compare the SAV3 concentrations measured. In this study, the highest SAV3 recovery and efficiency was made when combining electronegative filter with lysis buffer, by RT-ddPCR and RT-qPCR

analysis, with the former performing significantly better at higher dilutions. Following the *in vitro* study, a SAV3-cohabitant challenge trial using post-smolt Atlantic salmon, was carried out in order to evaluate the five concentration methods further. In this study, an electronegative filter combined with lysis buffer was the most suitable method for recovering SAV3 from seawater by RT-qPCR analysis. In addition, a positive correlation was found between SAV3 detections in cohabitant fish tissue and in water when using this concentration method. Further optimization and field testing of the filtration method for detection of SAV in seawater was with electronegative filter and elution with lysis buffer before sample analysis by RT-qPCR. Under the field conditions, early SAV-detections was made in seawater collected from inside net-pens compared to the monthly screening of fish. Higher SAV-recovery and early SAV detection were made in seawater compared to fish screening.

This new method could be a more straightforward, cost-efficient, time-saving, resource-saving, and not the least animal welfare-friendly approach for virus surveillance, with a potential for earlier implementation of disease control measures that may be applied to detect other fish pathogenic viruses than SAV. Moreover, it could also allow assessment of viral transmission and disease dynamics in fish farms.

Let's dive in!

Sammendrag

Den kommersielle akvakulturen vokser kontinuerlig med etterspørselen etter fisk og sjømatprodukter. I Norge utgjør 94% av det totale havbruket av atlantisk laks (*Salmo salar* L.), noe som gjør den til den viktigste arten i norsk havbruk. Med den høye etterspørselen etter fisk og fiskeprodukter globalt sett, fortsetter lakseoppdretten på en intensiv måte. Dette fører til at fisk konstant trues av å bli smittet og lide av forskjellige sykdommer. Pankreas sykdom (PD), som er forårsaket av *salmonid alphavirus* (SAV), er en systemisk sykdom som regnes som en av de mest alvorlige virussykdommene som også påvirker fiskevelferden negativt hos oppdrettslaksen. Det er også en av de mest økonomiskt viktige fiskesykdommene innen havbruk i Europa. Denne sykdommen ble først rapportert i 1976 i Skottland og på 1980-tallet i Norge, hvor den ble en meldepliktig sykdom (liste 3) i 2007.

I 2017 ble det nasjonale overvåkingsprogrammet for PD intensivert i Norge, med innføring av en PD-sone for SAV2 og SAV3, og to overvåkingssoner nord og sør/sør-øst om PD-sonen, for å redusere konsekvensene av sykdommen innenfor de definerte sonene, samt for å forhindre videre spredning av SAV. Dette overvåkingsprogrammet er avhengig av en tidkrevende og ressurskrevende tilnærming, som involverer månedlig prøvetaking av minst 20 fisk fra alle SAV-negative operative sjøvannsanlegg for salmonide fisker, og analyse av hjertevev fra hver av disse fisker ved RT-qPCR analyse.

Gjennom årene har det blitt gjort noen betydelige fremskritt i utviklingen av filtreringsmetoder for å konsentrere lave mengder patogener i vann, og overvåkingsprogrammer for forskjellige skadelige patogener er allerede etablert basert på denne teknikken. Dermed var fokuset for denne studien å utvikle og optimalisere en filtreringsmetode for påvisning av SAV i sjøvann, noe som gjør selektiv og invasiv tradisjonell testing av fisk overflødig.

Denne studien ble delt inn i tre trinn; opprinnelig testet *in vitro*, etterfulgt av evaluering i en smitteforsøksmodell, og til sist vurdert under feltforhold. *In vitro* studien ble utført for å teste fem kombinasjoner av to forskjellige elektroladet filtre, og fire forskjellige bufferløsninger for konsentrasjon og påvisning av SAV3 i sjøvann, ved å tilføye SAV3 i 1 liter kunstig og naturlig sjøvann. SAV3 ble kvantifisert ved bruk av RT-qPCR og RT-ddPCR for å sammenligne de målte konsentrasjonene. I denne studien ble de høyeste konsentrasjonene og effektiviteten av SAV3 funne når man kombinerte elektronegativt filter med lyseringsbuffer, ved RT-ddPCR og RT-qPCR-analyse, hvor den førstnevnte

presterte betydelig bedre ved høyere fortynninger. Etter *in vitro* studien ble det utført en SAV3-kohabitant smitteforsøk med post-smolt atlantisk laks for å evaluere de fem konsentrasjonsmetodene. Også i denne studien var elektronegativt filter kombinert med lyseringsbuffer den mest egnede metoden for å påvise SAV3 fra sjøvann ved bruk av RT-qPCR. I tillegg ble det funnet en positiv korrelasjon mellom SAV3-påvisninger i kohabitant fiskevev og i vann, ved bruk av denne konsentrasjonsmetoden. Ytterligere optimalisering og feltprøving av filtreringsmetoden for påvisning av SAV i sjøvann var med elektronegativt filter og eluering med lyseringsbuffer, før prøveanalyse ved RT-qPCR. Under feltforhold, ble tidlig SAV-påvisning gjort i sjøvann oppsamlet fra innsiden av merdkanten sammenlignet med månedlig screening av fisk. Det ble også avdekket høyere SAV-konsentrasjoner og effektivitet, og tidligere SAV-påvisning i vann sammenlignet med screeninganalyse av fiskevev.

Denne nye metoden kan være en mer rett frem, kostnadseffektiv, tidsbesparende, ressursbesparende og ikke minst dyrevelferdsvennlig tilnærming for virusovervåking, med potensial for tidligere implementering av sykdomskontrolltiltak og med mulighet for å oppdage andre fiskepatogene virus enn SAV. Videre kan det også tillate vurdering av virusoverføring og sykdomsdynamikk i oppdrettsanlegg.

La oss hoppe i det!

List of papers

Paper 1:

Development and evaluation of a method for concentration and detection of salmonid alphavirus from seawater

Weli, S.C.; Bernhardt, L-V.; Qviller, L.; Myrmel, M.; Lillehaug, A.

Published: Journal of Virological Methods, Volume 287, January 2021, p. 113990.

DOI: 10.1016/j.jviromet.2020.113990

Paper 2:

Concentration and detection of salmonid alphavirus in seawater during a post-smolt salmon (*Salmo salar*) cohabitant challenge

Bernhardt, L-V.; Myrmel, M.; Lillehaug, A.; Qviller, L.; Weli, S.C.

Published: Diseases of Aquatic Organisms, Volume 144, March 2021, p. 61-73

DOI: 10.3354/dao03572

Paper 3:

Early detection of salmonid alphavirus in seawater from marine farm sites of Atlantic salmon (*Salmo salar*)

Bernhardt, L-V.; Lillehaug, A.; Qviller, L.; Weli, S.C.; Grønneberg; E; Nilsen, H.; Myrmel, M.

Submitted: Diseases of Aquatic Organisms, February 2021

1. Introduction

1.1. Marine life

Our home planet Earth has existed for about 4.54 billion years (Patterson et al. 1955, Dalrymple 2001, USGS 2007). The earliest life forms we know of may have been based on primarily RNA, which seemingly preceded the life forms that are based on DNA genomes and protein enzymes (Robertson & Joyce 2012, Higgs & Lehman 2015). For this reason this hypothetical stage in the evolutionary history of life on Earth is commonly known as the "RNA world" (Robertson & Joyce 2012, Higgs & Lehman 2015). Nevertheless, recent research studies have shown that RNA and DNA may actually have coexisted before the emergence of the origin of life (Xu et al. 2019, 2020, Bhowmik & Krishnamurthy 2019).

Evidence shows that the emergence of the first life forms probably began in the oceans more than 3.7 billion years ago (Dodd et al. 2017) and developed into more complex life at least 636 million years ago (Gradstein et al. 2012). However, a more recent paper claims to have found macroscopic multicellular algae from up to 1.6 billion years ago, in the early Mesoproterozoic oceans (Zhu et al. 2016, Bengtson et al. 2017).

Today, the world's oceans cover approximately 70 % of the globe (Suttle 2007) and are homes to several different marine species, ranging from viruses to blue whales, in a vast diversity of habitats and environmental conditions.

1.2. Aquaculture

The term "aquaculture" refers to farming of aquatic organisms (i.e. fish, molluscs, and crustaceans) as well as aquatic plants and macroalgae in different water types (i.e. fresh, brackish, and marine waters) (FAO 2020). In this context, farming is a concept that comprises some intervention in the breeding process, in order to enhance production (FAO 2020). It is a thousand-year-old activity that has been evolving and advancing following farmers' curiosity and learning from errors and cooperation (FAO 2020). One of those advances was made in 1955, with the construction of a net-pen by Vik brothers for the purpose of farming fish (Hovland & Møller 2010, Myrseth 2020). Following this, the world's first Atlantic salmon (*Salmo salar* L.) farm was established in 1970 by the Grøntvedt brothers, when salmon smolts were placed in floating open net-pens off the island of Hitra, located on the west coast of Norway (Hovland & Møller 2010, Myrseth

2020). Thereby, the world's first successful generation of farmed Atlantic salmon was harvested in the early 1970s (Hovland & Møller 2010, Myrseth 2020). Ever since marine aquaculture have been proliferating, making Norway the world's second-largest exporter of fish and seafood in the world, after the oil and gas industry (Johansen et al. 2019).

Today, Norway is the leading salmon-producing nation in terms of market share, and the seafood industry is of high significance to the Norwegian economy (Johansen et al. 2019). Fish farms are located along Norway's western coastline, from the south (Agder) to the north (Finnmark) (Venvik 2005) and typically in floating open marine net-pens (**Figure 1**). To date, there are relatively few salmon producing countries globally. According to production, the five major salmon-producing countries of Atlantic salmon are Norway, Chile, United Kingdom, Canada, and the Faroe Islands, listed in descending order (Iversen et al. 2020).

In Norway, the Atlantic salmon (*Salmo salar* L.) comprises 94 % of the production of farmed fish (Directorate of Fisheries 2019a) and provide high-value nutrients that represent a valuable part of a healthy diet to humans (Khalili Tilami & Sampels 2018). This fish species originates from Norwegian waters and is an anadromous species, meaning it is born in freshwater but migrates to seawater later in life (Venvik 2005). Hatching and smolt production happens in land-based freshwater tanks, while the growth to market size happens in marine net-pens, as a way to take into account the natural lifecycle of the salmon (Venvik 2005). The brood stock is transferred to freshwater typically in autumn, followed by stripping of eggs and fertilization with milt (Jones 2009). Hatching takes place in freshwater when the fertilized eggs are around 500 degree days and become fry, which start feeding when they are around 850 degree days (Jones 2009). Thereafter, the feeding fry grow and become parr and further develops into smolts under temperature and light manipulation in freshwater tanks (Jones 2009). The smolts are transferred to sea sites typically in the spring the year following hatching, which is after 8-16 months when they weigh around 40-120 g (Jones 2009), and each farm sea site typically contain a single generation of fish (Jones 2009). The marine phase of Atlantic salmon farming lasts up to two years at the sea site (Jones 2009, Pettersen et al. 2015). The fish are then harvested when they weigh around 4-6 kg and transported by well-boat to the slaughterhouse (BarentsWatch 2021).

The domesticated species, such as the farm-raised Atlantic salmon, is the result of several generations of selective breeding and presents today a modified behaviour and

tolerance compared to the wild salmon (Glover et al. 2017). The breeding objective included growth performance initially but have gradually included more economically important traits, such as age at sexual maturation, resistance to furunculosis, resistance to infectious salmon anaemia (ISA), filet colour, fat content, fat distribution, growth in freshwater, body shape and resistance to infectious pancreatic necrosis (IPN) (Thodesen & Gjedrem 2006).

Figure 1. *Floating net-pens with Atlantic salmon (Salmo salar L.) at a marine farm site. Photo: L-V. Bernhardt.*

1.3. Safeguarding fish health

A healthy fish is a happy fish, and striving for good fish welfare in the aquaculture industry is beneficial for aquaculture sustainability and profit. Personnel management, biology, food and feeding, fish density, husbandry techniques, handling and transport, slaughter, and disease prevention could impact the farmed fish's welfare status (Segner et al. 2019). Therefore, improving the welfare of farmed fish could reduce the stress on the fish, which thereby becomes less susceptible to different diseases. One way is to use prophylaxis in aquaculture, which can enhance the fish performance and achieve improved product quality and production efficiency from more sustainable aquaculture (Gudding 2012, Bang Jensen et al. 2012, Gudding & Van Muiswinkel 2013).

When selecting sea sites, the species, culture method, salinity, water temperature, water flow and water-body exchange rates, feed type, stocking density, the hydrography of the site, the husbandry practices, the closeness to other farms, and local regulations are all factors that could impact the sustainability of the marine fish farming, and thus need

to be taken into account (Wu 1995, Jones 2009). Atlantic salmon grows best in sites with water temperatures ranging between 6-16 °C, salinities of 33-34‰ and with water flows sufficient to eliminate waste and to ensure optimal oxygen levels (~ 8 ppm) in water (Jones 2009).

Several factors can impact farmed fish husbandry. The rearing environment is one of them and involves, e.g. protecting the farmed fish from predators, preventing the fish from escaping and minimizing noise from pumps and other external disturbances (Segner et al. 2019). However, monitoring the water quality (e.g. temperature, pH and oxygen) at optimal levels is considered one of the most important techniques for fish welfare. After all, fish are in constant contact with water; thus, poor water quality or sudden changes in the water parameters can lead to acute and chronic health and welfare problems (Segner et al. 2019).

After each production cycle, fish are harvested for slaughter. The harvest, transport and slaughter involve many stressful steps that may harm fish welfare and product quality. Therefore, the duration and intensity of these steps should be reduced (Segner et al. 2019). Following slaughter, fallowing must take place at the site for at least two months before introducing a new generation of fish to the site (Lovdata 2008).

Whether the farming system is extensive or intensive can be determined by the stocking density (kg/fish/m^3), which gives information about the water volume the fish has available to move freely in (Segner et al. 2019). The stocking density can impact negatively on fish welfare (Turnbull et al. 2005), and should not exceed 25 kg/fish/m^3 for brood stock and farming of salmon and rainbow trout (with the exception for slaughter cages and closed production units), according to the aquaculture management regulations in Norway (Lovdata 2008).

Today, the growing demand for salmon to sustain human consumption leads to unsustainable farming with high-density populations that induce stress in the fish, making them more susceptible to various infectious production-related pathogens (Pettersen et al. 2016, Hoem & Tveten 2019). Thus, safeguarding fish welfare could be ensured by preventing spreading of the disease causing pathogen through early detection and sufficient and routine disinfection that could prevent any introduction of disease agents during transfer of infected fish, and the usage of contaminated equipment (Segner et al. 2019).

1.4. The health triangle

In the past, effective ways of the dealing with disease cause was centred mostly on the pathogen and sometimes its interaction with the host, while environment and farm management were largely neglected. However, the cause of disease needs to be broadly defined if recurrent disease outbreaks are to be controlled. For this reason, the health triangle (host, pathogen and environment) has been extremely useful to researchers in their attempts in understanding disease outbreaks (Snieszko 1974, Egger et al. 2003). The interrelationships between the host, pathogen and environment are presented in **Figure 2.** This interaction means that if the environmental conditions are favourable for the pathogen, then it could cause disease in the host (Snieszko 1974). In this context, a host could apply to the farmed fish, whereas the pathogen could be any microorganism (i.e. bacteria, virus, parasite or fungus) that causes the disease.

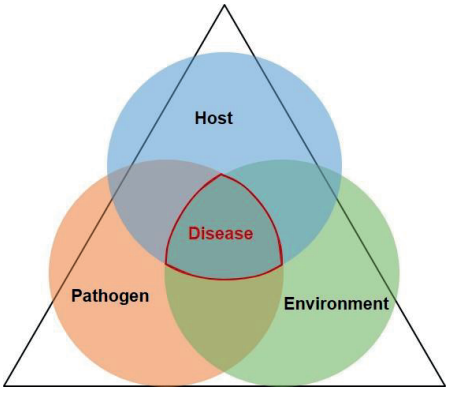

Figure 2. *The relationship between the host, pathogen and environment. Modified figure from Snieszko* (1974).

Opportunistic pathogens are non-specific but are characterized by taking advantage of atypical environmental stressors or a host experiencing impaired immune function, thereby becoming pathogenic (Gudding 2012). For the non-specific pathogens, however, it is not, e.g. stress and resilience to the infectious disease that are the important causes of the disease, but biosecurity, infection control, surveillance and combat (Lillehaug et al. 2015).

In a farming situation such as in aquaculture, the farmed fish are exposed to high levels of different environmental factors, i.e. stressors that can lead to a weakened

immune system (Segner et al. 2019, Hoem & Tveten 2019). Some of these stressors include poor feed quality, a high stocking density (Turnbull et al. 2005) and poor water quality (such as hypoxia and abnormal pH) (Lillehaug et al. 2015). The consequences include high availability of susceptible fish that become exploited to the very extreme by different pathogens that have adapted to their environment, facilitating rapid transmission of the infection (Segner et al. 2019, Hoem & Tveten 2019).

With measures to reduce stress induction on fish and by enabling early detection of the pathogen, actions could be taken to prevent the further spread on farms neighbouring to infected farms. This is beneficial both in terms of spread and economy for the industry as it reduces the consequences of the disease (Sommerset et al. 2021), including less medication and treatment, improved growth rate and food conversion, which ultimately lead to better product quality (Segner et al. 2019). It also emphasizes the importance of early biosecurity measures and regulations in terms of transport.

1.4.1. Control of diseases

Maintaining a sustainable aquaculture industry and achieving a good health status are fundamental issues for welfare reasons and can be done through disease control and disease prevention (Gudding 2012). The disease control measures are aimed at achieving successful fish health management and include good management practices and approved drugs and/or vaccines (Gudding 2012). The disease prevention in aquaculture is typically based on legislation and is primarily done through vaccination (Gudding 2012).

In Norway, immunoprophylaxis has been used for disease prevention for more than 40 years, namely, through vaccination with typically inactivated vaccines, and it is one of the reasons for the successful development of a sustainable fish-farming industry (Gudding 2012). Vaccines can stimulate the immune system of fish by developing an effective immunological mechanism, which can prevent the development of clinical diseases and thereby reduce the spread of infection (Gudding & Van Muiswinkel 2013).

In the Norwegian aquaculture industry, farmed fish are normally vaccinated against furunculosis (*Aeromonas salmonicida*), vibriosis (*Vibrio anguillarum*), cold water vibriosis (*Vibrio salmonicida*), winter ulcer (*Moritella viscosa*), IPN (*Infectious pancreatic necrosis virus*) and yersiniosis (Sommerset et al. 2020). A field study showed that vaccinations against pancreas disease (PD) have a positive effect by reducing the number of outbreaks, decreasing the cumulative mortality and decreasing the number of fish

discarded at slaughter (Bang Jensen et al. 2012). The most recent available vaccine against PD (*Salmonid alphavirus*) is a new monovalent vaccine based on DNA technology which was introduced around three years ago (Felleskatalogen 2021). Seemingly, this vaccine has a better effect in the field than any of the other available vaccines (Sommerset et al. 2020). However, most of the available PD vaccines are monovalent (Deperasińska et al. 2018), meaning that another vaccine will have to be used and protect against the other diseases mentioned above. Today, vaccinations against PD are carried out mostly within the PD zone (i.e. Western- and Mid-Norway) (Sommerset et al. 2020).

A good indicator of the occurrence of bacterial diseases is the usage of antibacterial agents (Gudding 2012). However, since the 1980s and early 1990s when all salmonid fish in Norway started being vaccinated against the most common bacterial diseases just before the seawater transfer, those vaccinations resulted in that most of the antibiotic prescriptions were made for non-specific bacterial infections (Lillehaug et al. 2018). Hence, within the Norwegian aquaculture industry today, there is an overall low antibiotic consumption (Sommerset et al. 2020), which has lead to a negligible development of antimicrobial resistance (Lillehaug et al. 2018).

For almost four decades, different kind of viruses have caused severe diseases in aquaculture, and the virus-related diseases are considered the major cause of the high mortality rates in the marine phase of the farmed Atlantic salmon (Kibenge 2016). During production in the seawater phase, significant losses of fish persist and threaten the industry's sustainability, and virus-related diseases are considered one of the major causes of the high mortality of Atlantic salmon (Sommerset et al. 2020).

Three viral diseases are dominating in Norwegian salmon aquaculture, and they are PD caused by SAV, Cardiomyopathy syndrome (CMS) caused by *Piscine myocarditis virus* (PMCV) and Heart and Skeletal Muscle Inflammation (HSMI) caused by *Piscine orthoreovirus 1* (PRV-1), ranged in descending order with regards to their incidence of disease outbreaks in 2019 (Sommerset et al. 2020). In addition, ISA caused by *Infectious salmon anaemia virus* (ISAV) is a contagious and serious viral disease causing high mortalities of farmed Atlantic salmon that have had an increased number of outbreaks in 2020 (Sommerset et al. 2021). The listed viral diseases in Norwegian aquaculture are PD, which is notifiable on a national level (list 3), and ISA, which is a non-exotic notifiable fish disease (list 2) (Sommerset et al. 2020).

Early detection of ISA and immediate removal of ISAV infected fish could allow successful combatting of disease outbreaks and prevent the further spread of the virus (Sommerset et al. 2020). Since 2015, the industry, fish health services and the Norwegian Food Safety Authority have been collaborating for systematic monitoring, which involves monthly inspections and sampling to detect ISA as early as possible in control zones created in the event of an ISA outbreak (Sommerset et al. 2020). This emphasizes the importance of early biosecurity measures and regulations in terms of transport.

In 2017, the national surveillance program for PD was intensified in Norway, thereby introducing a PD zone for the entire country and two national surveillance zones for SAV2 and SAV3 (Lovdata 2017). According to legislation introduced in 2017, monthly sampling of fish from all marine sites holding salmonid fish is required for extensive PCR screening for SAV. This is done in order to prevent the further spread of SAV. There are different strategies for reducing SAV infection depending on if the farmed fish is in any of the PD endemic zones or in an area that has been declared "SAV-free" (Lovdata 2017). Rapid slaughter of SAV infected populations followed by fallowing has been considered favourable both in economic terms and for prevention of SAV spread to a surveillance zone (Sommerset et al. 2020). Thus, a repeated PD outbreak at the same site is more likely to be caused by a new introduction of the agent than for it to be caused by a reinfection with SAV (Jansen et al. 2017). However, the legislation allows fish with positive SAV2 infections outside the PD zone to culture on till harvest, leading to further northerly spread of the infection (Sommerset et al. 2020). Thus, obligatory vaccination in this area might be able to reduce the infection pressure and the risk for further spread of the virus (Sommerset et al. 2020).

Preventing spread of infectious diseases in aquatic animals can also be achieved through surveillance, by using an animal-friendly technique that fulfils the 3Rs (Replacement, Reduction and Refinement) (Russell & Burch 1959, CIOMS 2012), making the sacrifice of the host redundant. This has been shown by previous research studies which used a water filtration technique which has shown potential for earlier implementation of disease control measures, as reported for, e.g. the oomycete *Aphanomyces astaci*, causing the crayfish plaque (Strand et al. 2011, 2014, 2019, Strand 2015, Rusch et al. 2020), the fish ectoparasite *Gyrodactylus salaris* (Rusch et al. 2018) and SAV (Andersen et al. 2010, Jarungsriapisit et al. 2016a,b, 2020).

1.5. Salmonid alphavirus (SAV)

Microorganisms constitute more than 90 % of the living biomass in the world's oceans, of which viruses comprise only 15 % due to their small size (Suttle 2007). Nevertheless, every second, virus cause approximately 10^{23} virus infections in the world's oceans (Suttle 2007).

Viruses are by far the most abundant "life forms" in the aquatic environments, with each litre of natural seawater found to contain up to 250 billion virus particles (Bergh et al. 1989). The world's oceans are known to contain almost 200 000 different virus species (Gregory et al. 2019). Although these studies focused on DNA viruses, there are diverse RNA viruses as well in the marine systems (Lang et al. 2009). In fact, RNA viruses (single- and double-stranded, positive- and negative-sense) can infect a diverse range of host species and cause diseases that have devastating effects on the aquatic animal populations (Lang et al. 2009).

When it comes to SAV, it is a single-stranded RNA virus that causes PD which is a severe disease that typically affects farmed salmonid fish in the marine phase (Strauss & Strauss 1994, Graham et al. 2007, McLoughlin & Graham 2007, Deperasińska et al. 2018). In addition, it is a virus that has been classified by the World Organisation for Animal Health (OIE) as a notifiable pathogen in fish (OIE 2019).

1.5.1. Characteristics

SAV belongs to the genus *Alphavirus,* which is one of two genera of the family *Togaviridae* (Nelson et al. 1995, Weston et al. 2002). Two alphaviruses are known to infect aquatic animals; SAV and southern elephant seal virus (SESV) (Shi et al. 2018). However, SAV was the first alphavirus to be isolated from fish (Weston et al. 1999).

SAV is a lipid enveloped, positive-sense single-stranded RNA (+ssRNA) virus with an icosahedral single nucleocapsid that encloses a spherical shaped genome (Strauss & Strauss 1994, Deperasińska et al. 2018), measuring a diameter of approximately 60-70 nm and a total genome length of approximately 12 kilobases (kb) (Weaver & Frolov 2010, Taksdal & Sindre 2016). The SAV genome is composed of two open reading frames (ORFs); one encodes eight proteins, of which four are structural capsid glycoproteins (E1, E2, E3, and 6K) by the 3' end, while the other encodes four nonstructural proteins (nsP1-nsP4) by the 5' end and makes two-thirds of the genome (Strauss & Strauss 1994) (**Figure 3**).

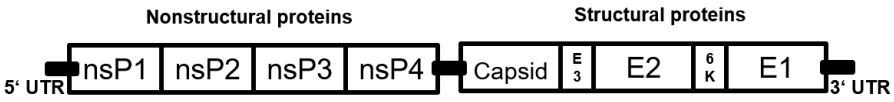

Figure 3. *The genome organization of SAV. Modified figure from Hodneland et al.* (2006).

Based on phylogenetic analysis of nucleotide sequences of the partial E2 gene and nsP3 gene of SAV from farmed Atlantic salmon and rainbow trout (*Oncorhynchus mykiss*), this virus species was divided into six subtypes (i.e. SAV1-SAV6) (Fringuelli et al. 2008). However, in 2020 it was reported about a seventh subtype (SAV7) which was isolated in non-salmonid fish (i.e. *Labrus begylta*), and found to be both genetically and phenotypically distinct from the other known SAV genotypes (Tighe et al. 2020).

In Norway, there are currently two known SAV subtypes (i.e. SAV2 and SAV3), which are more closely related genotypes than the other subtypes (Fringuelli et al. 2008, Tighe et al. 2020). These are forming two separate PD endemic zones with SAV3 in South-western Norway and SAV2 in North- and Mid-Norway (Hjortaas et al. 2016, Sommerset et al. 2020) (**Figure 4**).

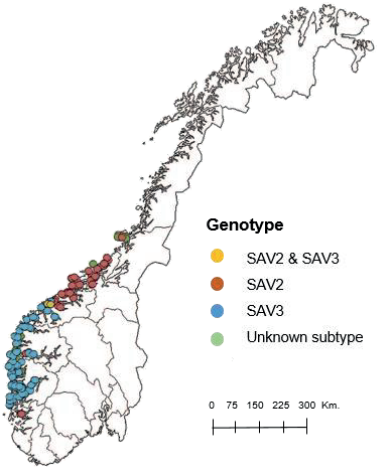

Figure 4. *The map shows outbreaks of pancreas disease (PD) in Norway in 2019 caused by either SAV2, SAV3, or an unknown subtype. Modified figure from the Fish health report 2019* (Sommerset et al. 2020).

The first appearance of SAV3 was made in the late 1980s in Norway (Poppe et al. 1989). SAV3 has not been identified in any other countries and was the only genotype detected in Norway for more than two decades. However, in 2010, marine SAV2 was reported for the first time in Norway following a single introduction into Norwegian aquaculture (Hjortaas et al. 2016). One important difference in the clinical manifestation of SAV3 and marine SAV2, is that SAV3 infection typically causes higher mortality in Atlantic salmon compared to infection with the marine SAV2 (Taksdal et al. 2015). Furthermore, SAV3 has been detected in Atlantic salmon and rainbow trout (*Oncorhynchus mykiss*, Walbaum) only in seawater (Christie et al. 1998, Hodneland et al. 2005), while SAV2 has been detected in farmed Atlantic salmon in both seawater (Hjortaas et al. 2013) and freshwater (Hjortaas et al. 2016), and in farmed rainbow trout in freshwater (Graham et al. 2003). Additionally, SAV2 has also been detected in Arctic char (*Salvelinus alpinus*) in freshwater (Lewisch et al. 2018).

The SAV infection in fish likely occurs through gills, skin or intestine, whereas the main target cells for SAV are still unknown (Jansen et al. 2017). During the SAV infection, viraemia occurs first, followed by clinical signs and histopathological changes associated with PD (McLoughlin & Graham 2007). A positive correlation has been found between viraemia and virus shedding (Andersen et al. 2010, Graham et al. 2011). Detection of SAV has been made in serum (Graham et al. 2010), pseudobranch, gill, heart (atrium and ventricle), (head) kidney, pancreas (pyloric caeca with associated pancreatic tissue), somatic muscle and brain, by RT-qPCR (Andersen et al. 2007). When comparing SAV3 and SAV2, the former subtype presents more pronounced histopathology, especially in the cardiac and pancreatic tissue, than the latter subtype, shown by another study (Graham et al. 2011).

Infected farmed salmonid fish are the main reservoir of SAV (Jansen et al. 2017). However, the natural reservoir of SAV is unknown, but it might be the wild fish as SAV RNA has been detected in internal organs of different flatfish species (Snow et al. 2010). Previous research has also shown an abundant genetic diversity within subtypes in both farmed and wild fish, and that various SAV subtypes can occur on the same fish farm (Gallagher et al. 2020).

Replication cycle

For the most part, alphaviruses are arthropod-borne viruses, replicating in both invertebrate (e.g. mosquitoes or other hematophagous arthropods serving as vectors) and vertebrate hosts, thereby causing a persistent lifelong infection and acute and usually short-lived infection, respectively (Strauss & Strauss 1994). Alphaviruses replicate within the cytoplasm of the host cell, and progeny virions bud through the plasma membrane during the replication cycle, as explained by Herath et al. (2016) and Leung et al. (2011) in **Figure 5**. In this way, they introduce new genetic information into the organism and yield progeny virus that ultimately drives the evolution (Shi et al. 2018). SAV has been detected in sea lice (*Lepeophtheirus salmonis*) by RT-PCR analysis (Petterson et al. 2009). Regardless, this virus does not seem to need vectors (OIE 2019).

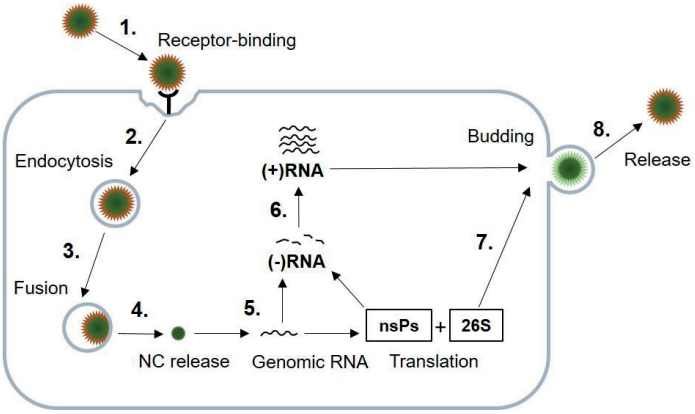

Figure 5. *The virus-host cell interaction during the replication cycle of alphavirus.* ***(1)*** *The E2 envelope protein of the alphavirus binds to the host cell's receptors.* ***(2)*** *Thereafter, the virus enters the host cell's cytoplasm via clathrin-mediated endocytosis.* ***(3)*** *Due to the low pH, the viral envelope and the endosome fuses in the endosome.* ***(4)*** *The virus releases its nuclear material, i.e. the nucleocapsid (NC), into the cytoplasm.* ***(5)*** *After disassembly, the +ssRNA genome is released for translation and cleavage. The genomic RNA, i.e. two-thirds of the genome from the 5' end, is translated into non-structural proteins (nsPs), while the subgenomic RNA is translated into structural proteins (26S).* ***(6)*** *The complementary minus-strand from the nsP, serves as a template for the production of genomic and subgenomic RNA, while* ***(7)*** *26S travels towards the plasma membrane, encapsulates the genomic RNA and buds out from the host cell, thereby* ***(8)*** *acquiring an external lipid envelope before release. Modified figure from Leung et al.* (2011).

1.6. Pancreas disease (PD)

The first report of PD was from Scotland in 1976 (Munro et al. 1984), followed by Ireland in the early 1990s (Rodger et al. 1992). A decade later, it was first reported in Norway in 1989 (Poppe et al. 1989), where it is considered as one of the most severe farm-related virus diseases (Jansen et al. 2015, Sommerset et al. 2020). The aetiological agent of this disease was previously called *salmon pancreas disease virus* (SPDV) (Nelson et al. 1995). However, the name *salmonid alphavirus* (SAV) was proposed when Weston et al. (2002) found out that SPDV and *sleeping disease virus* (SDV) are isolates of the same virus species.

1.6.1. Characteristics

PD has been observed in European farmed salmonids in both seawater and freshwater (Jansen et al. 2017). This disease has a systemic manifestation that typically affects Atlantic salmon in the seawater phase, during their first or second year after seawater transfer (Hodneland & Endresen 2006).

The clinical signs associated with PD include lethargy, loss of appetite, failure to grow, abnormal swimming behaviour and varying mortality (McLoughlin et al. 2002) (**Figure 6**). However, salmonids can be infected with SAV without actually developing the associated disease itself, or they could have PD without presenting any clinical signs or mortality (Graham et al. 2006).

Histopathological changes characteristic of PD include degenerations in the pancreas and in the cardiac and skeletal muscle tissues (Nelson et al. 1995, McLoughlin et al. 1996, 2002, Taksdal et al. 2015, Jansen et al. 2017). Degeneration of oesophageal muscle has also been observed (Ferguson et al. 1986a,b). Typical gross signs include empty intestines, yellow mucoid gut contents (i.e. faecal casts), and petechial haemorrhages in the pyloric caecal fat (Nelson et al. 1995, McLoughlin et al. 2002).

Recovery from PD can take several weeks or months (McVicar 1987). However, previous research states that following a PD outbreak, 15 % of the fish surviving from PD have difficulty growing properly, and these fish become so-called “runts” (McLoughlin & Graham 2007). Survivals may even die some months after the primary outbreak (Munro et al. 1984). Hence, the disease poses negative impacts on fish welfare and causes a significant economic loss to the aquaculture industry (Aunsmo et al. 2010).

Figure 6. *PD-affected Atlantic salmon swimming close to the water's surface and crowding in the corner of a net-pen at a marine farm site. Photo: L-V. Bernhardt.*

1.7. The role of transmission

"Yesterday upon the stair
I saw a man who wasn't there
He wasn't there again today
Oh how I wish he'd go away."
- William Hughes Mearns (1875–1965)

SAV spread mainly through horizontal transmission, via water contact, which has been supported by several different studies (e.g. McLoughlin et al. 1996, Andersen et al. 2010, Kongtorp et al. 2010, Graham et al. 2011, 2012, Xu et al. 2012, Stene et al. 2016, Jarungsriapisit et al. 2016b). The horizontal transmission occurs mainly passively due to water currents and wind (Lillehaug et al. 2015, Stene et al. 2016) or actively via human activity, vectors or fomites, causing sporadic outbreaks, and this is supported by several epidemiological studies (Kristoffersen et al. 2009, Aldrin et al. 2010, 2015, Jansen et al. 2017). Vectors that may increase the risk of the virus spreading in fish farms include, e.g. well-boats, cargo vessels and service personnel arriving with service boats (Murray & Peeler 2005, Lillehaug et al. 2015, Tadaishi et al. 2016).

The majority (95 %) of farms experiencing PD outbreaks in Norway have been found to have had an outbreak previously in the past year and to be within the distance of 50 km from a PD identified fish farm (Aldrin et al. 2010). The probability of horizontal transmission is reduced with increasing distance between fish farms (Kristoffersen et al. 2009, Aldrin et al. 2010, 2015). Based on this, at least one SAV infected fish farm could pose a potential source of infection, and with decreasing distance to the infectious farm, the risk for SAV to be introduced to a neighbouring farm will be increasing (Aldrin et al. 2010).

Recent experimental trials demonstrated the detection of SAV shedding from Atlantic salmon into seawater (Andersen et al. 2010, Jarungsriapisit et al. 2016a,b) and their viability and infectivity (Jarungsriapisit et al. 2020). SAV RNA has also been detected in organic matter such as faecal casts and mucus, suggesting that the virus particles spread in a non-homogenous manner (Graham et al. 2011, 2012). Moreover, accumulated levels of SAV RNA have been found in the lipid layer on the seawater surface (probably due to fat leaking from decomposing dead PD-affected farmed Atlantic salmon), which could be a cause of long-distance spread with the help of the wind and ocean currents (Stene et al. 2016).

It has been reported that SAV can survive for extended periods in the aquatic environment and that it is associated with an increased risk of spreading, especially in the marine phase (Graham et al. 2007). In addition, a reduced survival time can be seen at higher temperatures for SAV in seawater (Jarungsriapisit et al. 2020) and in the presence of organic matter (Graham et al. 2007). Moreover, these are factors to why PD could be considered one of the most challenging farm-related virus diseases to control and eradicate.

In terms of reducing the spread of SAV and the consequences of PD outbreaks, the following implementations on practical biosecurity measures have been suggested: general good hygiene including regular cleaning of tanks and net-pens, earlier slaughter of the fish to prevent further virus spread, removing dead fish, segregating fish generations, arranging the fish to get through periods with increased risk of a PD outbreak without becoming sick, careful handling of the fish, and overall reducing the stress exposure, providing a feeding strategy that strengthens the immune system of the fish so that the fish can fight against the disease more effectively and control of parasites and other pathogens (Lillehaug et al. 2015, OIE 2019). In addition, avoiding, e.g. movement of

live fish, sharing of fish farming equipment and personnel between fish farms and effective disinfection procedures of e.g. well-boats and aquaculture equipment, might also contribute to the prevention of SAV transmission from infected to non-infected fish populations (Kristoffersen et al. 2009, Lillehaug et al. 2015).

1.8. Methods for detecting the presence of SAV

"An ounce of prevention is better than a pound of cure"
- Benjamin Franklin (1706-1790)

1.8.1. Diagnostic methods

The "gold standard" is considered the traditional approach, including the best single diagnostic test or a combination of tests that have been thoroughly tested (Parikh et al. 2008). It is the current recommended method in the field for verification of a particular disease or for detection of the disease-causing pathogen (Parikh et al. 2008). The value of a screening test is that it aims at identifying asymptomatic individuals who may have an infection, in contrast to a diagnostic test which aims at determining the presence or absence of disease (Parikh et al. 2008). Thus, a positive screening test should be followed up by a diagnostic test in order to establish a definitive diagnosis in a population (Parikh et al. 2008).

OIE (2019, 2021) has defined criteria for targeted surveillance and diagnosis of SAV infection and evaluates the following methods: gross signs (**Figure 7**), histopathology (**Figure 8**), immunohistochemistry (**Figure 8**), isolation of SAV in cell culture, serum neutralization assay, reverse-transcription quantitative real-time polymerase chain reaction (RT-qPCR) and reverse transcription PCR (RT-PCR) with sequencing (**Table 1**).

SAV infected fish populations can stay SAV-positive until slaughter (Jansen et al. 2010a,b), while lifelong persistent infection on an individual level has not been reported (OIE 2019). Fish that are sampled in the early stages of SAV infection might present very mild or no histopathological degenerations in their skeletal muscles, while in mid- to late-stage infection, the pancreatic and cardiac tissue changes in several fish examined will be in recovery (McLoughlin & Graham 2007). However, it can also be the case that fish individuals are infected with SAV and test positive without actually developing the

associated disease (Graham et al. 2006). Therefore, the timing of the infection stage is seemingly crucial in terms of using fish tissues to screen for SAV in fish farms.

In order to confirm the presence of SAV, two independent laboratory tests such as microscopic pathology, cell culture, RT-PCR, or serology need to be performed (OIE 2019). For confirming a diagnosis of SAV infection, there are some methods that are recommended, some that are considered standard methods, some that are methods that could be applied in some situations but with some limitations (e.g. cost and accuracy) and then there are some that are not recommended (OIE 2019) (**Table 1**).

Table 1. *The methods currently available and acceptable for targeted surveillance and diagnosis of infection with SAV in adult fish, with different levels of availability, utility, and diagnostic specificity and sensitivity. Modified table from OIE* (2019).

Method	**Suspected case**	**Confirmed case**
Gross signs		
Histopathology		
Immunohistochemistry		
Isolation in cell culture		
Serum neutralization assay		
RT-qPCR		
RT-PCR with sequencing		

Note: Green = recommended method; yellow = standard method; orange = has application in some situations but with some limitations (e.g. cost and accuracy); red = currently not recommended.

Clinical signs and gross pathological changes associated with PD (Ferguson et al. 1986a,b, Nelson et al. 1995, McLoughlin et al. 1996, 2002, Graham et al. 2006, Taksdal et al. 2015, Jansen et al. 2017) are considered non-pathognomonic (OIE 2019). Therefore, laboratory diagnostic tests need to be applied in order to verify a diagnosis (McLoughlin & Graham 2007). The traditional and recommended analysis methods for surveillance and/or diagnosis of SAV/PD in populations of Atlantic salmon have been presented in **Table 2**.

Table 2. *Traditional and recommended analysis methods for surveillance and/or diagnosis of SAV/PD, to differentiate between infected and assumed "SAV-free" test populations of Atlantic salmon. Modified table from OIE* (2019, 2021)*.*

Methods	Identification	Test purpose	Reference
Histopathology	Gill, heart, pyloric caeca and the associated pancreatic tissue, liver, kidney, spleen and skeletal muscle (red and white), and skin with associated skeletal muscle (red and white) at the lateral line level.		Nelson et al. 1995 McLoughlin et al. 1996 McLoughlin et al. 2002 Graham et al. 2006 Taksdal et al. 2015 Jansen et al. 2017 Ferguson et al. 1986a,b
OIE recommended organs:	Heart and mid-kidney	Diagnosis	Jansen et al. 2019
Detection of anti-SAV antibodies (in cell culture)	Susceptible cell lines: e.g. CHSE-214*, RTG-2, BF-2, SHK-1, EPC, CHH-1.		Graham et al. 2008 Hodneland et al. 2005 Karlsen et al. 2006 Nelson et al. 1995 McLoughlin & Graham 2007 Herath et al. 2009 Jansen et al. 2010a,b Taksdal et al. 2015
OIE recommended organs:	Heart and mid-kidney	Diagnosis	Jansen et al. 2019 Hall et al. 2014
Detection of SAV molecular biological techniques	RT-PCR RT-qPCR Genotyping by sequencing		Shi et al. 2017 Hodneland & Endresen 2006 Gallagher et al. 2020 Jansen et al. 2010a,b
OIE recommended organs:	Heart and mid-kidney	Surveillance / Diagnosis	Jansen et al. 2019
Detection of neutralizing activity against SAV	Serum or plasma	Surveillance	Jansen et al. 2019

** Commonly used for primary SAV isolation.*

Note: BF-2 = Bluegill Fry-2; CHSE-214 = CHinook Salmon Embryo - 214; CHH-1 = CHum Salmon Heart-1 cells; EPC = Epithelioma papulosum cyprinid; RTG-2 = Rainbow trout gonad – 2; SHK-1 = Salmon Head Kidney-1.

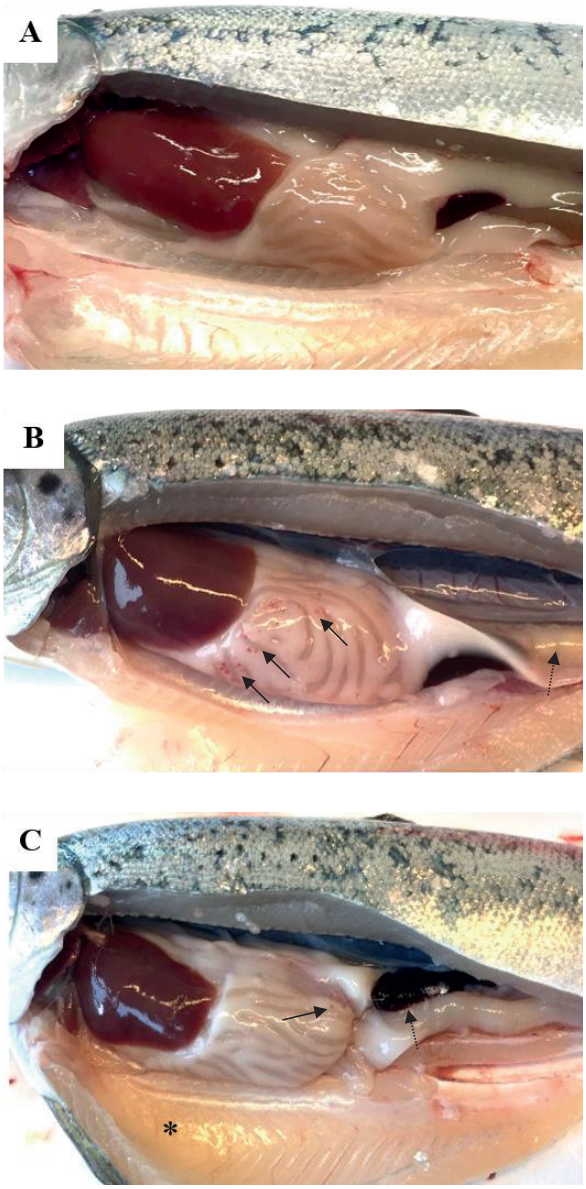

Figure 7. *Gross pathological lesions in three different cohabitant fish from an experimental SAV infection (**Paper II**). **(A)** Normal fish. **(B)** Fish with PD associated signs: Petechial haemorrhagia on adipose tissue surrounding pyloric caeca (—▶) and yellow mucoid intestinal content (⋯▶). **(C)** Fish with PD associated signs: Ascites (*), congestive splenomegaly (⋯▶) and petechial haemorrhagia in adipose tissue surrounding pyloric caeca (—▶). Photo: L-V. Bernhardt.*

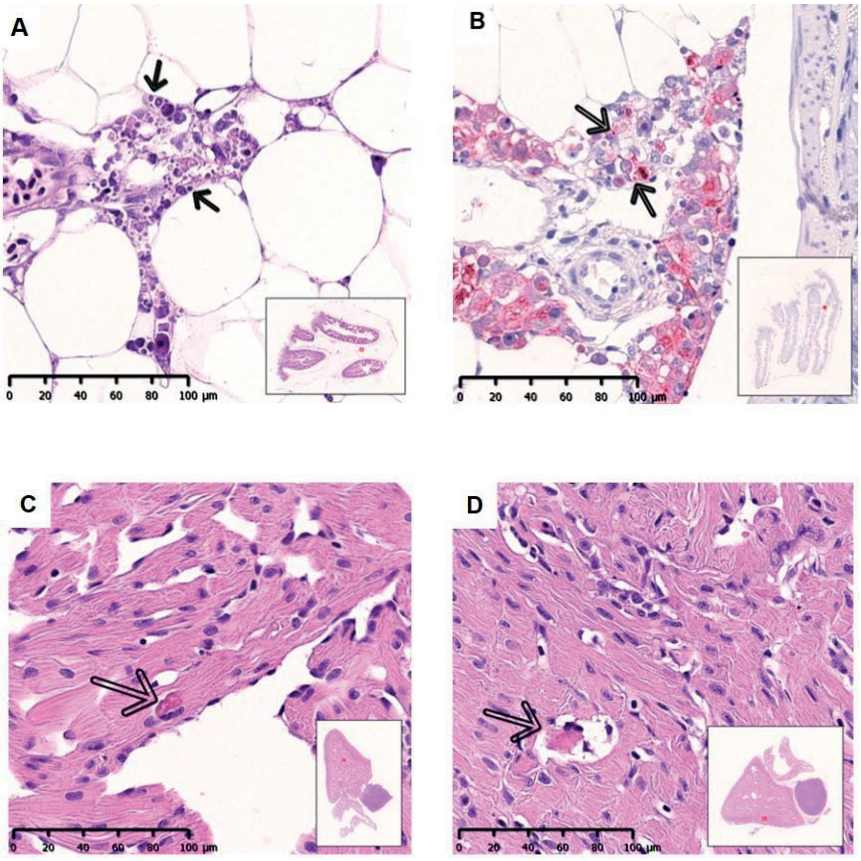

Figure 8. *Histopathological lesions in pancreas and heart tissue from cohabitant fish from an experimental SAV infection (**Paper II**).* ***(A)*** *Pancreas. H&E, 20x. Necrosis and severe loss of exocrine pancreatic tissue. Some of the pyknotic exocrine cells have been marked by arrows (⟶).* ***(B)*** *Pancreas. IHC, 20x. Positive IHC-staining in the exocrine pancreatic cells, characterized by a distinct red colouring of the cytoplasm of the exocrine pancreatic cells. Some of the positive IHC-stained exocrine pancreatic cells have been marked by arrows (⟶).* ***(C)*** *Heart. H&E, 20x. Affected cardiomyocytes manifest shrunken cytoplasm, with an intense eosin staining and loss of striations and pyknotic nuclei, characterized as focal myocardial degeneration, which have been marked by arrows in the spongy cardiac ventricular layer (⟶).* ***(D)*** *Heart. H&E, 20x. Focal myocardial degeneration have been marked by arrows in the compact cardiac ventricular muscle layer (⟶). Photo: L-V. Bernhardt.*

The most apparent differential diagnoses to PD are CMS and HSMI, and they commonly cause severe myocarditis associated with myocardial necrosis in different heart regions in addition to causing changes in the pancreas and skeletal muscle tissues (Ferguson et al. 1990, McLoughlin et al. 2002, Kongtorp et al. 2004). Another severe viral disease of salmonid fish resulting in pancreatic lesions is the IPN (Smail et al. 2006, Ellis et al. 2010) (**Table 3**).

Table 3. *The pattern of the different histopathological changes associated with infection with SAV, HSMI, CMS and IPN. Modified table from OIE* (2019).

Fish organ tissue	Infection with SAV	HSMI	CMS	IPN
Heart	+ *	+ *	+ **	-
Pancreas	+	-	-	+
Skeletal muscle	+	+	-	-

** The compact layer of the ventricle is more severely affected.*

*** Mainly the inner spongious layer of the ventricle and the atrium.*

1.9. The surveillance program for SAV/PD in Norway

In 2007, PD became a national notifiable fish disease (list 3) in Norway, meaning that detection of SAV needs to be followed by control measures. Following this, a national regulation was introduced in order to prevent the further spread of SAV, thereby preventing, limiting, and combatting PD in defined zones throughout Norway. Revision of the regulations in 2017 for the entire country involved introducing a PD zone which is where the disease is endemic for SAV2 and SAV3 (i.e. Western- and Mid-Norway), and two national surveillance zones north and south/south-east of the PD zone (Lovdata 2017). The national surveillance zones could either be free from PD or comprised of control areas consisting of two local surveillance zones (Lovdata 2017). The local surveillance zones consist of both a combatting zone and a surveillance zone, and they are intended to be kept free from SAV (Lovdata 2017).

Surveillance through monitoring of SAV is an essential issue in the health management of the farmed Norwegian Atlantic salmon. With measures to reduce stress induction on fish and by enabling early detection of the pathogen, actions could be taken to prevent the further spread on farms neighbouring to infected farms. The surveillance program for SAV/PD in Norway requires sampling of fish and subsequent testing for the

virus at least once a month from all SAV-negative marine operative farm sites with Atlantic salmon, rainbow trout and Arctic char (Lovdata 2017). This is done in order to reduce the consequences of the disease within the defined PD zones, as well as to prevent further spread of SAV to a surveillance zone (Lovdata 2017). A minimum of 20 fish from each of these sites needs to be sampled for PCR-analysis every month until SAV is eventually detected at the site or until the fish population is slaughtered (Lovdata 2017). When fish are meant to be moved from a site, at least 60 fish from this site need to be sampled and tested within the last three weeks before the move (except for fish that are meant to be moved to slaughterhouses). Sampled fish could include dead brood stock if considered suitable for sampling (Lovdata 2017).

An SAV-positive identification of one or more fish from a site by PCR, results in the whole site receiving a "PD suspected" status according to the Norwegian Food Safety Authority (Mattilsynet 2018). However, in order to confirm the PD diagnosis, additional fish must be sampled for further analysis (Lovdata 2017). This means that organs from at least 10 fish that are both considered susceptible to PD and the most likely to reveal any occurrence of PD must be sampled for further analysis by PCR, cell culture and histopathology (Lovdata 2017). All samples should be sent to the national reference laboratory (i.e. Norwegian Veterinary Institute, NVI) together with information about the vaccine status (Lovdata 2017). If this first step of fish sample analysis is unable to confirm PD, extended sampling and testing may be carried out (Lovdata 2017). However, if the second analysis step is unable to reveal any new SAV-positive samples, then the "PD-suspected" status can be invalidated (Lovdata 2017). This means, for the PD diagnosis to be confirmed, SAV-positive PCR results, clinical symptoms and signs associated with PD, including gross pathology and histopathology, are all required to be present (Lovdata 2017).

2. Knowledge gaps

At the initiation of this study, which began in 2017, Norway had just revised a regulation to reduce the consequences of PD within the defined PD zones and to prevent further spread of SAV (Lovdata 2017). The surveillance program for SAV/PD is both resource-demanding and time-consuming as it requires monthly and invasive sampling of at least 20 fish from every marine operative farm site of salmonids for further examination in order to confirm the presence of SAV infection (Lovdata 2017).

Several studies supported that SAV spread mainly through horizontal transmission (McLoughlin et al. 1996, Andersen et al. 2010, Kongtorp et al. 2010, Graham et al. 2011, 2012, Xu et al. 2012, Stene et al. 2016, Jarungsriapisit et al. 2016b). Epidemiological studies also confirmed that SAV mainly spread passively via water, causing sporadic PD outbreaks (Kristoffersen et al. 2009, Aldrin et al. 2010, 2015, Jansen et al. 2017). A majority (95 %) of the PD outbreaks in Norway were even found to have had an outbreak previously in the past year (Aldrin et al. 2010), and neighbouring infected fish farms were shown to pose a potential source of infection (Aldrin et al. 2010).

At the beginning of this study, four experimental studies investigated the detection of SAV in water, of which three were successful (Andersen et al. 2010, Jarungsriapisit et al. 2016a,b), while one was not (Graham et al. 2011). Although, Graham et al. (2011) did not manage to detect SAV in water, they did reveal that SAV might spread homogenously via mucus and faeces which was also shown by another study that following year (Graham et al. 2012). Between 2010 and 2016, several studies revealed that SAV could be transmitted between net-pens within sites and between sites with the help of the wind and ocean currents (Kristoffersen et al. 2009, Aldrin et al. 2010, Lillehaug et al. 2015, Stene et al. 2016), which further confirmed the role of water in the horizontal transmission of SAV (Kristoffersen et al. 2009, Aldrin et al. 2010, Lillehaug et al. 2015, Stene et al. 2016).

Andersen et al. (2010) was first to demonstrate SAV3 shedding during infection and showed that the virus could be detected from seawater using a water filtration method. The method was based on a modified virus adsorption-elution (VIRADEL) technique which also proved successful in a subsequent study for detection of SAV3 RNA and viable virus particles from seawater (Jarungsriapisit et al. 2020).

Following the new surveillance program for SAV/PD, thousands of fish are sacrificed every year for analysis through a selective and invasive approach that relies on

analyzing a relatively small number of fish that should represent the whole population at a farm site (Lovdata 2017). However, when SAV is recently introduced into a population, the chances of sampling infected fish are small, given that there are probably few infected individuals. This gave us reason to believe that filtering water for surveillance of SAV, shed from the infected fish into their aquatic environment, could serve as a more straightforward, cost-efficient, time-saving, resource-saving, and not the least animal welfare-friendly method alternative for surveillance of SAV infection in farm sites with fish population.

The following knowledge gaps were identified and needed to be filled:

- Which combination of filter and buffer solution is the best for concentrating and detecting SAV in seawater?
- Is there a correlation between the detection of virus shedding into the water and the detection of SAV in the fish organ tissues traditionally sampled for surveillance?
- Could water sampling from a fish population's aquatic environment give a more representative image of the SAV status and allow earlier detection of the virus than sampling of relatively few fish individuals in connection with the mandatory surveillance program for SAV/PD?
- Can filtration of seawater samples be applied as an alternative method for the surveillance of SAV in fish populations under natural field conditions?

3. Aims of this study

The background of this PhD study is the prospect of an alternative to the traditional selective and invasive testing of fish. Thus, the working hypothesis of this study is that a filtration method for detecting SAV in seawater collected from farm sites with SAV infected salmonid fish may serve as a straightforward, cost-efficient, time-saving, resource-saving, and not the least animal welfare-friendly method. This was investigated through the development, optimization and field-testing of a new filtration method for revealing the presence of SAV shed from infected fish into their aquatic environment. The overarching research question of the study was: What potential does the water filtration method have in surveilling for SAV in seawater at farm sites of Atlantic salmon? Thus, this study had the following aims and sub aims:

1. An *in vitro* study to test different concentration methods, which involve five different combinations of two different electrocharged filters and four different buffer solutions for concentration and detection of SAV3 from SAV3-spiked artificial and natural seawater (**Paper I**). This was done in order to:
    a. find out which filter-buffer combination gives the highest SAV3 recovery efficiency when filtering seawater.
    b. determine the limit of detection (LOD) and limit of quantification (LOQ) for SAV3 in seawater when using the most suitable concentration method, with RT-qPCR and RT-ddPCR assays.

2. An experimental study to evaluate the different concentration methods during a SAV3 cohabitant challenge trial with post-smolt Atlantic salmon by filtration of tank water samples for concentration and detection of SAV3 (**Paper II**). This was done in order to:
    a. determine the most suitable concentration method, i.e. with the overall highest and most consistent SAV3 recovery.
    b. determine the LOD and LOQ with RT-qPCR when using the most suitable concentration method.
    c. determine whether the SAV3 concentrations detected in seawater were related to the SAV3 concentrations detected in fish organ tissues.

3. A field study to assess the filtration method for detecting SAV in seawater, collected monthly for the surveillance purpose, from several different Norwegian marine farm sites of Atlantic salmon within the SAV3 endemic area (i.e. Western- and Mid-Norway) (**Paper III**). This was done in order to:
    d. investigate if the water filtration method could be applied as an alternative method for surveillance of SAV at marine farm sites with Atlantic salmon.
    e. investigate if SAV detection could be made earlier in seawater compared to the screening of fish.

4. Summary of papers I-III

Paper I - Development and evaluation of a method for concentration and detection of salmonid alphavirus from seawater.

Weli, S.C.; Bernhardt, L-V.; Myrmel, M.; Qviller, L.; Lillehaug, A.

https://doi.org/10.1016/j.jviromet.2020.113990

Currently, the prevalence of salmonid alphavirus (SAV) in Norwegian Atlantic salmon farms is largely surveyed via monthly sampling of at least 20 fish from each of all "SAV-free" marine farm sites of salmonids. This results in the sacrifice of thousands of fish every year and is an invasive, selective, time-consuming, and resource-demanding approach that relies on analyzing a relatively small number of fish that should represent the whole fish population on a site.

This study is a first step towards developing and optimizing an alternative method for surveillance of SAV by using a more cost-efficient, straightforward, resource-saving, time-saving, and animal welfare-friendly approach based on the detection of SAV in seawater. Five different concentration methods involving two different electro-charged filters and four different buffer solutions were evaluated for the concentration of SAV subtype 3 (SAV3) from seawater. To test these, we spiked SAV3 into 1 L of artificial and natural seawater, which were filtered through the electrocharged filter followed by elution of the adsorbed material from the filter with either of four different buffer solutions. The SAV3 was quantified by using RT-qPCR and RT-ddPCR.

In this study, we found out that the combination of an electronegative membrane filter (MF-Millipore™ 0.45 µm MCE membrane, Merck Millipore, USA) with NucliSENS® Lysis Buffer (easy MAG®, bioMérieux, Marcy l'Etoile, France) gave the highest SAV3 recovery of 39.5 ± 1.8 % by RT-ddPCR, and 25.9 ± 5.7 % by RT-qPCR. Additionally, the limit of quantification (LOQ) and the limit of detection (LOD) in 1 L natural seawater were estimated to be 5180 and 200 SAV3 RNA copies L^{-1}, respectively, by RT-ddPCR. Moreover, LOD in 1 L natural seawater was estimated as 42 SAV3 RNA copies L^{-1} by RT-qPCR. Based on these results, it was suggested that the electronegative filter combined with lysis buffer would be a candidate for further validation in an experimental trial.

Paper II - Concentration and detection of salmonid alphavirus in seawater during a post-smolt salmon (*Salmo salar*) cohabitant challenge

Bernhardt, L-V.; Myrmel, M.; Lillehaug, A.; Qviller, L.; Weli, S.C.

https://doi.org/10.3354/dao03572

This study followed our previously described *in vitro* study (**Paper I**) and was performed experimentally to investigate further the filtration method's ability to detect SAV3 directly from seawater. This was done by testing the five concentration methods during a six-week cohabitant challenge trial, using post-smolt Atlantic salmon at the Industrial and Aquatic Laboratory (ILAB) in Bergen, Norway. Shedder fish were intraperitoneally (i.p) injected with either a low SAV3 dose of $2 \cdot 10^2$ $TCID_{50}$ $fish^{-1}$, a high SAV3 dose of $2 \cdot 10^4$ $TCID_{50}$ $fish^{-1}$, or a virus-free Leibovitz-15 (L-15) cell culture medium containing 2% FBS (mock inoculum) and transferred into three 500 L tanks. Samples of 1 L tank water and cohabitant fish organ tissues (mid-kidney and heart) were collected together, from all three tanks at 16 different time points.

SAV3 was first detected in tank water (7 days post-challenge, DPC) and later in cohabitant fish organ tissue samples (12 DPC). The concentration method involving the electronegative filter combined with lysis buffer presented the highest SAV3-recovery and was therefore considered the most suitable method. This conclusion is also consistent with the above-mentioned *in vitro* study. The SAV3 was quantified by RT-qPCR, and the LOD and LOQ, when using the most suitable concentration method, were found to be 902 and 2736 SAV3 RNA copies L^{-1}, respectively. A significant positive correlation was found between SAV3 detected in the tank water concentrates, and SAV3 detected in the mid-kidney samples.

Based on these results, we suggest that the most suitable concentration method (i.e. electronegative filter combined with lysis buffer) is tested for surveillance of farmed salmonid populations, as a part of a biosecurity plan, for SAV under natural field conditions. The availability of such a water filtration method may result in earlier identification of SAV infection in a fish population and prevention of further virus spread between neighbouring farms. This could enable earlier disease control measures to improve the virological safety of the water environment.

Paper III - Early detection of salmonid alphavirus in seawater from marine farm sites of Atlantic salmon (*Salmo salar*)

Bernhardt, L-V.; Lillehaug, A.; Qviller, L.; Weli, S.C.; Grønneberg, E.; Nilsen, H.; Myrmel, M.

In this study, the water filtration method developed by our previous studies (**Paper I & II**) was applied to natural field conditions. Initially, a pilot study was performed at a PD diagnosed net-pen to determine the highest SAV concentrations in seawater samples collected at three different water depths (0.15 m, 5 m, and 10 m) and from three different sampling points (two inside and one outside the net-pen). The most suitable but yet practical approach was thereafter applied to a large-scale field study, which was performed over the study period May 2019 - January 2020. Seven different marine farm sites with Atlantic salmon located in the SAV3 endemic zones along the western Norwegian coast and with no initial suspicion of SAV infection were collected for seawater samples. Seawater samples were collected from the top layer inside each net-pen at the marine farm site in parallel with the ongoing mandatory surveillance program for SAV/PD. The filtration method used here involved filtration through an electronegative filter, followed by rinsing with lysis buffer, before the RNA extraction and analysis by RT-qPCR.

The pilot study showed that the highest SAV concentrations were concentrated and detected from the top layer inside the net-pen. Using this strategy, SAV from seawater was detected in all sites and at an earlier stage in all sites, where the fish tested positive for SAV, compared to traditional sampling strategies. Namely, a significant negative temporal relationship was observed at all sites, meaning that the fewer SAV copies there were in the seawater, the more days it took until SAV was detected in the fish samples. Based on these findings, it was shown that the water filtration method has a great potential to be implemented for surveillance of farmed salmonid populations for early detection of SAV infection.

5. Methodological considerations

This PhD study's objective was framed with a focus on developing and optimizing a filtration method for detecting SAV in seawater at Atlantic salmon farm sites. In order to do this, a three-step methodological approach was considered, as follows:

1. An *in vitro* study involving SAV3-spiking of artificial and natural seawater and analysis by RT-qPCR and RT-ddPCR (**Paper I**).
2. An experimental study involving a six-week SAV3 cohabitant challenge trial of post-smolt Atlantic salmon (*Salmo salar* L.) (**Paper II**).
3. A field study performed for a study period from May 2019 to January 2020 in a study area inside the SAV3 endemic zone, including seven different marine farm sites of Atlantic salmon (**Paper III**).

5.1. Study designs

The steps towards developing and optimizing a filtration method for the detection of SAV in seawater were done by defining the design, evaluating the method's limitations and suitability for its intended purpose, and optimizing the procedures. There are different methods used for concentrating viruses from water, and these depend on different factors, including, e.g. the water volume, the water type, the filter type (e.g. material, diameter and pore size), the eluent (i.e. buffer solution) used for re-dissolving the filtrate, the extraction of nucleic acids and the PCR method.

Finding the most suitable strategy can be complex as it depends on the purpose, objectives and goals, and how these goals should be delivered will be influenced by the circumstances and conditions of the study (**Table 4**). In this study, a combination of an *in vitro* study, a challenge trial and a field study was the strategy used to investigate the effectiveness and sensitivity of a new water filtration method for detecting the presence of SAV shed from infected fish into their aquatic environment.

Table 4. *Recommended data collection and approach for water filtration studies. Inspiration and modification of table from Goldberg et al.* (2016).

Stage	Information
Design	Presence/absence, quantity and/or viability of the target species.
Water collection	Registration form, water volume, type of water (i.e. seawater or freshwater), number of samples (incl. negative control), sampling approach (i.e. water depth, sampling point), and site.
Concentration method	Method, filter type (i.e. composition, diameter, pore size), eluent type, target species, and filtering location.
Sample preservation	The transport media, storage temperature and duration should be considered depending on whether the filtration was performed onsite or offsite.
Extraction	Method, eluate volume, contamination precaution incl. negative control.
Analysis	Method, positive criteria: technical replicates (and their interpretation) and repeated sampling/analysis, primer/probe amplicon length, master mix, positive & negative controls, internal process control (IPC), inhibition detection & handling, reaction concentration & thermal profile, standard curve preparation & quality (RT-qPCR).
Sequencing	Separation of SAV subtypes (SAV1-SAV7).
Transportation	Registration form (see **Appendix**) with information about when/where/how the study was performed and of whom. Each sample should be labelled with an identification label (Bartram et al. 1996).

5.1.1. The *In vitro* model

In vitro is Latin for "in glass", meaning it happens in an artificial environment, outside the living organism (Merriam Webster 1828). It is a technique performed in a controlled environment (i.e. laboratory) that is rapid, cost-effective, and convenient. It can also provide crucial information for further method development and implementation without testing in fish. For these reasons, it is useful as a preliminary screening method. The first step towards finding the most suitable filter-buffer combination in terms of giving a high SAV recovery and efficiency in seawater was performed in the laboratory (**Paper I**).

Two different electrocharged filters (electronegative and electropositive) and four different buffer solutions were combined as five different concentration methods and tested for their ability to concentrate SAV3 from 1 L seawater (**Paper I**). Both artificial seawater (Sigma-Aldrich, Germany) and natural seawater were spiked with a known amount of cell-cultivated SAV3.

5.1.2. The challenge model

The cohabitant challenge trial served as a representative model by simulating the virus's spread and thereby achieving a natural exposure route to the cohabitant fish. With this model, an overview of the entire study population was assessed, and this allowed for monitoring of the PD progression from the moment it began and more closely studying the SAV route of transmission, as opposed to what the *in vitro* study was capable of.

The SAV3 cohabitation challenge trial was carried out for six weeks in tanks provided by ILAB in Bergen, Norway (**Paper II**). Atlantic salmon post-smolts were used in the challenge, which took place approximately one year after hatching and was approved and performed according to the Norwegian Animal Research Authority (NARA) and animal welfare regulations (FOR-2015-06-18-761).

In this study, three separate 500 L tanks were used, including one high viral dose tank, T_H, one low viral dose tank, T_L, and one negative control tank, T_C. The SAV3 inoculum used was based on pooled heart and head kidney samples of Atlantic salmon from verified PD cases in the Hordaland region of Norway (Taksdal et al. 2015). Shedder fish were i.p. injected with either a low SAV3 dose, a high SAV3 dose or a mock inoculum. The seawater samples were collected first, followed by the fish sampling, as explained in **Figure 9**, and the sampling was performed randomly at 16 sampling time-points through the challenge.

Each tank contained 55 cohabitants and 15 shedder fish, meaning the initial stocking density was 15.5 kg $m^{-3,}$ and the initial proportion with shedder fish was 21 % (15/70 fish) which is within the range of previous studies of SAV (e.g. Graham et al. 2011, Taksdal et al. 2015) and also in line with the 3Rs (Russell & Burch 1959, CIOMS 2012).

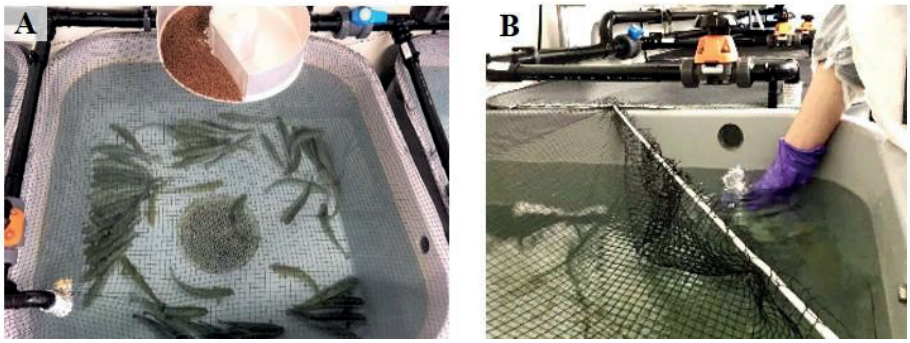

Figure 9. *Seawater sampling during the SAV3 cohabitant challenge trial.* ***(A)*** *A 500 L tank with seawater entering from the inflow pipe visualized in the lower-left corner (water flow rate ~ 950 L* h^{-1} *tank*$^{-1}$*). The outflow was carried out by suction through a drain, which can be seen in the tank bottom.* ***(B)*** *The seawater sampling was always performed from the lower right corner of the tank by submerging the bottle ~ 0.15 m below the water surface with the mouth of the bottle turned towards the water current. Photo: L-V. Bernhardt.*

5.1.3. The field model

Following the challenge trial, the most suitable concentration method was further evaluated in the natural field conditions in order to assess the water filtration method as an alternative to fish screening for surveillance of SAV in fish farms. A field study could uncover the applicability of the method under realistic farming conditions and the environmental factors that were not immediately obvious during either the *in vitro* study or the experimental study. Environmental factors include, e.g. variation in water temperature, water quality, stocking density, the movement of live fish in and between farms, slaughters, biosecurity measures, personnel management, but also the water currents and weather conditions (e.g. wind) could influence the dispersion and dilution of SAV shed from infected fish (Kristoffersen et al. 2009, Aldrin et al. 2010, 2015, Lillehaug et al. 2015, Stene et al. 2016, Jansen et al. 2017).

The first approach was based on a pilot study designed to find out at which water depth and sampling point the highest SAV concentrations could be found in seawater to optimize the water filtration method for the following main field study (**Paper III**). The pilot study was performed for a duration of one day in August 2018. It was located at a PD diagnosed site with Atlantic salmon which had received a commercially available SAV vaccine. This site was located within the SAV3 endemic zone (i.e. Western Norway) and

duplicate seawater samples were collected from one of its net-pens at three different water depths from three different sampling points, two inside the net-pen as shown in **Figure 10** and one outside the net-pen.

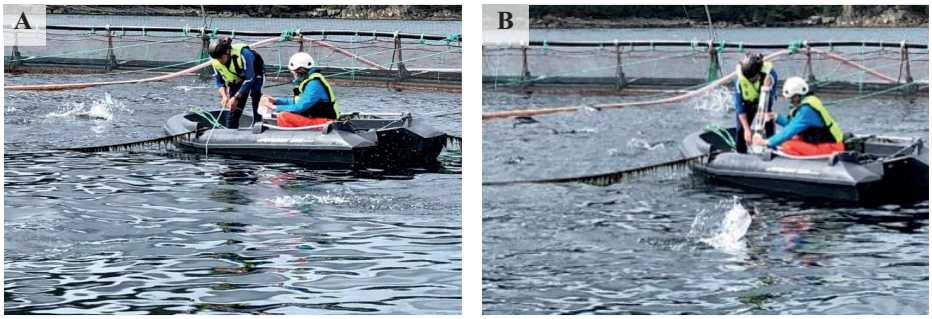

Figure 10. *Pilot study. Seawater sampling was carried out from a rubber dinghy inside the net-pen. Samples from 5 m and 10 m water depths were collected by using a Ruttner 2 L Standard Water Sampler, which was lowered into the water depths (A) and emptied into a sterilized 1 L plastic bottle (B) as visualized. Photo: L-V. Bernhardt.*

Following the pilot study, the main field study was performed involving seven Norwegian marine farm sites of Atlantic salmon (with no initial suspicion of SAV infection), located within the SAV3 endemic zone in the Western Norwegian coastline, i.e. in the counties of Vestland (formerly Hordaland) and Rogaland (**Paper III**). These sites were selected based on their recorded positive PD history in earlier fish generations during the years 2013-2018 (records from https://www.barentswatch.no/fiskehelse). All sites had received a commercially available SAV vaccine. The anonymity of all involved farm sites was a prerequisite for research collaboration.

The main field study was initiated by a visit and was carried out between May 2019 and January 2020, in connection with the surveillance program for SAV/PD. Seawater samples were thereby collected consistently from all net-pens at each site, approximately 0.15 m below the water surface, close to each net-pen's inner side, as shown in **Figure 11**. This sampling was performed by a team of fish health inspectors, with each inspector assigned their own specific task. Before the start of the field study, all involved fish health inspectors had received on-site instructions and educational material, including a manual,

instructional video recording and equipment, ensuring consistency in the way the seawater samples were collected and stored.

Fish were sampled (≥ 20 fish/site) in connection with the monthly sampling to comply with the mandatory surveillance program (according to the regulations) and thus, no additional fish were sampled for the field study. The fish samples collected at the seven sites were processed and analyzed for SAV by RT-qPCR at external laboratories. There was no information about which net-pen the sampled fish were withdrawn from, and sometimes occasional slaughters at selected net-pens and occasional transfer of the fish populations occurred between net-pens on the site due to e.g. treatment, during the study. For these reasons, both the seawater- and fish analysis were assessed at the site level.

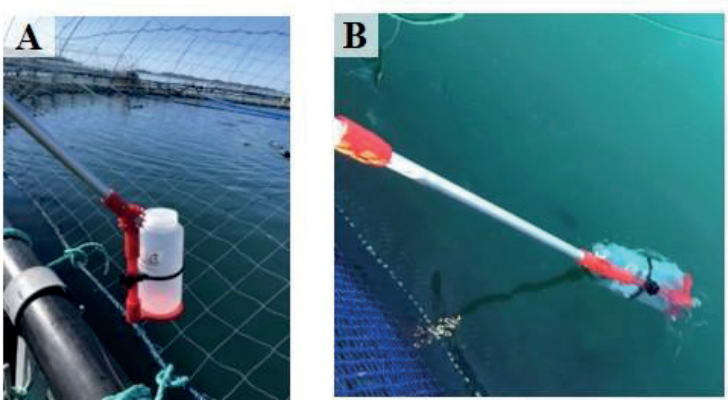

Figure 11. *Main field study.* ***(A)*** *A 1 L sterilized plastic bottle attached to a telescopic swing sampler (Bürkle GmbH, Germany).* ***(B)*** *Seawater sampling was performed by the inner side of the net-pen just below the water surface. Photo: L-V. Bernhardt.*

For each sampled site, a registration form was filled in by the fish health inspector and added to each shipment (see **Appendix**). The seawater samples from the main field study (**Paper III**) were stored with cooling elements in a cooling bag, as shown in **Figure 12,** and shipped by express delivery to the laboratory of NVI (Oslo, Norway), where the filtration took place within 24 h.

Figure 12. *Transportation of water samples during the main field study.* ***(A)*** *For each sampled site, bottles were stored wrapped with bubble wrap insulation, surrounded by cooling elements in a 45 L Proxon cooler box (Pinnacle, Tokyo Plast International Ltd., India).* ***(B)*** *A filled out registration form (**Appendix**) was added to each shipment and* ***(C)*** *shipped together with the water samples to the laboratory of NVI (Oslo, Norway). Photo: L-V. Bernhardt.*

5.2. Water filtration methods

The water collection approach and preservation of the water sample are factors that could affect the maximum recovery, and they are dependent on the water system and target species (Goldberg et al. 2016). Furthermore, with regards to choosing a suitable filter, different factors that include the type of filter, the filter's composition, diameter and pore size, should be considered based on the purpose of its application (Goldberg et al. 2016).

5.2.1. Water volume

Previous research recommends larger volumes and more water samples to detect the presence of the target species when the concentrations are expected to be low in the water (Strand et al. 2014, 2019, Rusch et al. 2018, 2020). These studies aimed at detecting different types of parasitic pathogens (*A. astaci* and *G. salaris*) in river systems with a density different from this study, which aimed at detecting virus from seawater collected at farm sites of fish populations where the pathogen is expected to be very much higher in comparison.

The volume size of choice for the water samples in this study was 1 L. Filtration of 1 L water samples for the detection of SAV from seawater has been shown successful by previous studies (Andersen et al. 2010, Jarungsriapisit et al. 2016a,b, 2020). Filtering larger volumes than 1 L, might increase the risk of inhibition during PCR due to the presence of PCR inhibitors (e.g. salt and organic matter) in the water (Strand et al. 2011).

Larger volumes and/or a high content of organic matter in the water can clog the filter; thus, it might be necessary to change the filter after filtering a smaller volume or using larger pore size filters. However, with larger pore size, the target species may not be captured and could therefore be lost during filtration.

5.2.2. Concentration methods

Concentrating the virus that have been eluted from the filter is one of the most important steps when aiming to achieve a high recovery from water samples (Gibson & Borchardt 2016). The reason for this is that the virus concentration in water is expected to be very low; thus, the concentration step is essential in terms of avoiding false-negative results when the water samples are analyzed by molecular methods (Gibson & Borchardt 2016).

Filter type

Filtering water for the concentration of pathogens began already in 1892 with the discovery of the first virus, i.e. Tobacco Mosaic Virus (TMV), which became the first "filterable virus" using a sterilizing filter and remained infectious thereafter (Rifkind & Freeman 2005). In 1910, poliovirus (*Enterovirus C*) was reported filterable and infectious following filtration of processed mucosa of the mouth and nose through a Berkefeld filter (Flexner & Lewis 1910).

Over the years the development progressed and a technique widely known as VIRADEL (virus adsorption-elution) which was used in this study was developed in the late 1960s (Wallis & Melnick 1967). This technique involves adsorption of viral particles to a surface by charge interaction and subsequent elution from that surface by a pH-adjusted solution (Wallis & Melnick 1967). This simple and efficient technique has been described by several different studies previously (Wallis et al. 1972, Farrah et al. 1976, Goyal & Gerba 1983) and has also been found successful for detection of SAV from seawater (Andersen et al. 2010, Jarungsriapisit et al. 2016a,b, 2020, **Paper I**, **II & III**).

In the 1970s, a study showed the importance of electrostatic forces in virus recovery from water when using microporous filters in VIRADEL methods (Sobsey & Jones 1979). There are two types of filters commonly used to adsorb virus from water, i.e. electronegative filters (MF-) and electropositive filters (MDS+) (Cashdollar & Wymer 2013). In the present study, both these microporous filter were used to filter the seawater

for the detection of SAV. Microporous filters can be categorized into three different types, i.e. depth, surface and screen (Merck 2021).

The MF- filter used in this study is a screen filter, characterized by its uniform structure, which enables all particles that are larger than the pore size of 0.45 µm to be retained on the surface, i.e. like a sieve (Merck 2021). The MDS+ filter is a depth filter, characterized by matrix made of compressed materials that enable particles to hold on to the filter by random adsorption or entrapping (Merck 2021). MDS+ filters have been used successfully before for filtration of seawater for SAV detection in different experimental studies (Andersen et al. 2010, Jarungsriapisit et al. 2016a,b), as shown in **Table 5**. The MF- filter has never been reported for detection of SAV in seawater before this study. However, this filter type has previously been used for the detection of poliovirus from seawater (Katayama et al. 2002).

Viruses carry a pH-dependent surface electrostatic charge in polar media such as water, and this electrostatic charge influences the virus' sorption processes (Michen & Graule 2010). The isoelectric point (pl) is defined as the pH at which the colloid carries a surface net charge of zero (Parks 1965), i.e. above the pl the net charge is negative and below the pl the net charge is positive.

In general, electropositive filters do not require preconditioning, but has the disadvantages that they can be expensive and typically clogs in more turbid waters (Cashdollar & Wymer 2013). Electronegative filters, however, are economical and are capable of filtering large water volumes even in more turbid water before clogging, but typically require preconditioning (Cashdollar & Wymer 2013). However, in this study, no preconditioning of the water sample or the filter was performed before filtration through the MF- filter (**Paper I, II & III**). For SAV to adsorb to MF- filter, the preconditioning step was probably not necessary in this study. The reason for this could be that SAV has a pl of 9.95 (Pickett et al. 2012) thereby making it positively charged, assuming that the pH of its environmental seawater chemistry is below the pl of SAV.

Other filtration techniques for virus detection from water could involve using glass wool filters, NanoCeram® filters or ultrafiltration. In the early 1990s, glass wool filters became a popular tool to concentrate viruses from large water volumes (Cashdollar & Wymer 2013). They are inexpensive and do not require preconditioning because of their electropositive charge (Cashdollar & Wymer 2013). However, these filters are packed by hand, resulting in great variations in the filter's performance (Cashdollar & Wymer 2013).

Another inexpensive electropositive filter is the NanoCeram® filter which has electropositive charge, does not require preconditioning and is an economic alternative to the 1 MDS electropositive filter. The NanoCeram® was reported in 2009 to have comparable or higher recoveries of Poliovirus and Norwalk virus from tap- and river waters than the 1MDS filter, suggesting that the NanoCeram® filters could be used as alternatives to 1MDS filters for routine viral monitoring of water (Karim et al. 2009).

For the past two decades, ultrafiltration has grown in popularity for concentrating viruses from large water volumes (Cashdollar & Wymer 2013, Strand et al. 2014, Borgmästars et al. 2017, Bisseux et al. 2018, Gerba et al. 2018), and it has also been used successfully to monitor the aquatic environment for harmful pathogens (Strand et al. 2014). This kind of filtration technique either makes the sample pass through the ultrafilter once (i.e. dead end) or through multiple circulations (i.e. tangential flow) (Cashdollar & Wymer 2013). It is an inexpensive alternative for recovering viruses from water; however, it has some major drawbacks, including its slow filtration rate and tendency for clogging in more turbid waters (Cashdollar & Wymer 2013).

Eluent type

Previous experimental studies have successfully eluted SAV-adsorbed MDS+ filter with either lysis buffer (Andersen et al. 2010) or L-15 medium (Jarungsriapisit et al. 2016a) as shown in **Table 5**. In this study, either of four different buffer solutions was used to rinse the filter (MF- or MDS+), including lysis buffer, 1 mM NaOH (pH 9.5), L-15 medium (pH 9.0) or L-15 medium (pH 7.3–7.75) (**Paper I & II, Table 5**). These four different buffer solutions have different properties. NaOH is usually used to adjust the pH value of a culture medium, affects the physical appearance of the culture media, and optimizes its ability to grow microorganisms. The L-15 (Leibovitz) medium contains phenol red, sodium pyruvate, and L-glutamine that all together are formulated for use in carbon dioxide-free systems that require sodium bicarbonate supplement, supporting, e.g. established cell lines. With regards to lysis buffer and extraction buffer, which were used in the field study (**Paper III**), they both contain chaotropic agents (i.e. guanidine thiocyanate and Triton X-100) that can break open the virus particle, meaning the virus might not be viable in cell culture after exposure (Ngo et al. 2017).

5.2.3. RNA extraction

Regardless of the water volume, filter type, and buffer solution used for concentrating the virus from water, the natural seawater samples will either way contain substances (e.g. salt and organic matter), that might affect the concentration and extraction of the virus nucleic acids and/or inhibit nucleic acid amplification. Detection of viruses in water by using a qPCR-based method which was the case in this study, consists of three steps which are: (1) concentration of the viruses, (2) extraction of the RNA, and (3) performance of RT-qPCR (Haramoto et al. 2018). Any of these three steps may mask the presence of a virus by a false-negative interpretation of the results and therefore, it is essential to have a good understanding in their functions and features, in order to verify failed methods (Diez-Valcarce et al. 2011).

Extraction platforms

The SAV RNA from the water concentrates in this study (**Paper I, II & III**) were extracted by using the easyMAG® robot (bioMérieux, Marcy l'Etoile, France). The easyMAG platform is a well-established platform considered a gold standard for efficient extraction of DNA and RNA due to its ability for silica to bind to the nucleic acids in high salt concentrations (Boom et al. 1990). It has also been used successfully for extraction for different types of RNA-viruses from water samples (Petterson et al. 2015) as well as for SAV RNA from fish tissue samples (Jansen et al. 2010a, 2019). An alternative platform that could be considered is the MagNA Pure which has shown high sensitivity and specificity comparable to easyMAG for extraction of viral RNA and DNA from different sample types (Verheyen et al. 2012, Edelmann et al. 2013, Hindiyeh et al. 2019) and has also been used in a previous study for extraction of SAV RNA from fish tissue samples (Robinson et al. 2020).

The SAV RNA extraction of tissue samples was performed by using another extraction platform called QIAcube® (Qiagen®, Germany) (**Paper II**) due to practical reasons. This platform has previously been used by relevant studies which extracted fish tissue samples for SAV detection (e.g. Hodneland et al. 2005, Graham et al. 2011, Hjortaas et al. 2016).

The eluate volume for the seawater concentrates was chosen based on how concentrated they were expected to be. For seawater collected *in vitro* and during the challenge trial, the initial SAV3 dose used to spike the seawater (**Paper I**) and inject the

shedder fish (**Paper II**) were known and expected to be higher than the SAV-concentrations in the seawater samples collected under natural field conditions (**Paper III**). For this reason, a higher eluate volume was chosen for the seawater samples in **Paper I & II** (i.e. 50 μl) than in **Paper III** (i.e. 40 μl). The decrease in the eluate volume ensured a more concentrated RNA eluate but might have increased the amount of natural RT-qPCR inhibitors that are expected to influence the RNA-virus quantification.

5.2.4. SAV-detection

Environmental samples such as natural water samples are prone to false-negative results, which can be caused by various factors, including poor RNA recovery (during filtration and after the nucleic acid extraction and purification steps), small amounts of template RNA in the sample, and PCR inhibitors (Goldberg et al. 2016). In this study, monitoring for the presence of SAV in water samples was done mainly by RT-qPCR, which enables high performance in the detection of waterborne viruses at low concentrations (Girones et al. 2010, Rački et al. 2014a,b). Nevertheless, a check of inhibition was made for all water samples in this study. With regards to PCR inhibitors, they can either be endogenous (i.e. derived from the water sample) or exogenous (e.g. introduced during sample processing or nucleic acid extraction). Natural seawater contains both salts and other PCR-inhibitors, which may have an influence on the RNA-virus quantification.

The PCR inhibitors can be removed by diluting the sample used in this study. It is a technique that reduces the inhibitory effects of the PCR inhibitors, which can reduce polymerase activities and binding of primers, and reduce the sensitivity of detection. However, the dilution method can also co-dilute the target RNA, resulting in non-detections (Tsai & Olson 1992). Another strategy is to analyze by RT-ddPCR (**Paper I**), which is less sensitive to any effect of inhibitors compared to RT-qPCR and does not require a standard curve to quantify the amounts of SAV RNA in the water samples (Rački et al. 2014a,b). Although RT-ddPCR is a more expensive method for testing only a few samples, the samples could then be run undiluted and without the need for a standard curve, thereby making it a quicker and more cost-effective procedure compared to RT-qPCR (Nathan et al. 2014), provided that RT-ddPCR has been sufficiently optimized for its purpose. Nevertheless, RT-qPCR is a powerful tool commonly used to detect and quantify SAV due to its high sensitivity and high specificity and has been recommended for detection of SAV by a recent field evaluation study (Jansen et al. 2019). RT-qPCR is also a

recommended method by the OIE for the surveillance and diagnosis of SAV/PD (OIE 2019). Additionally, it has been used with success in several different experimental studies for monitoring water samples for the presence of SAV by RT-qPCR (Andersen et al. 2010, Jarungsriapisit et al. 2016a,b, 2020). For these reasons, RT-qPCR was considered over RT-ddPCR in **Paper II & III**.

RT-qPCR assays

The Q_nsP1 assay is recommended for the detection of SAV by RT-qPCR analysis (OIE 2019) and was used to detect SAV in all the seawater samples throughout this study (**Paper I, II & III**). This broad-spectrum PCR assay was developed by Hodneland et al. (2006) and provided a rapid, sensitive, and specific detection method for SAV, when using RT-qPCR (Shi et al. 2017). It includes a primer pair and probe that together amplifies a region of 107 bp in the 5' end of the nsP1-gene, for all available SAV sequences (Hodneland & Endresen 2006).

The purpose of the analyses, i.e. whether they are for large-scale PCR reactions or for achieving higher throughput, will influence the master mix of choice. Initially, the Brilliant III Ultra-Fast qPCR Master mix was tested for SAV detection in the water samples by RT-qPCR. However, since it was found to not handle the PCR-inhibitors in the seawater samples satisfactory (unpublished data), an inhibitor-resistant RT-qPCR master mix such as TaqMan Fast Virus 1-Step Master Mix was considered instead (**Paper I, II & III**). It has previously been shown to enhance virus detection in water samples and to provide reliable, high sensitivity, high efficiency and consistent results for RT-qPCR from water samples (Borgmästars et al. 2017).

Further confirmation of SAV and the subtype detected in the seawater samples by RT-qPCR analysis in the field study (**Paper III**) was done through sequencing of the E2 gene in accordance with the standard protocol of NVI (Oslo, Norway). Another possibility could be to only sequence the nsP3 gene, but that would only have enabled confirmation of SAV but not revealed its subtype.

5.2.5. Process controls

Process controls are essential components for detecting viruses in food (Diez-Valcarce et al. 2011) and in water samples (Parshionikar et al. 2004, Gibson & Borchardt 2016, Haramoto et al. 2018). There are no established standards for acceptable values of virus

recovery and/or extraction-RT-qPCR efficiency in water samples (Haramoto et al. 2018). However, three types of process controls can be added into the sample to increase the accuracy during the interpretation of the observed data, either (1) before viruses are concentrated (i.e. whole-process control), (2) before the nucleic acid extraction (i.e. molecular process control) or (3) before the (RT)-qPCR processes (i.e. RT-qPCR control) (Hoorfar et al. 2004, Haramoto et al. 2018).

Previous research studies have studied SAV detection in seawater and shown success by using different process controls. One of these studies used *Halobacterium salinarum* and the aquatic rhabdovirus *Viral Haemorrhagic Septicaemia Virus* (VHSV) as a filtration control and an RNA extraction control, respectively (Andersen et al. 2010). Another study used *nodavirus* to validate the recovery from the RNA extraction and the subsequent cDNA synthesis (Jarungsriapisit et al. 2020).

In the field study (**Paper III**), *Mengovirus* strain MC_0 (type strain: ATCC VR-1957) was used as an internal process control (IPC) by adding a known quantity of this virus to each 1 L seawater sample before filtration (**Figure 13**). Although there are no ISO criteria for SAV detection in water, a suitable process control would likely be one that is similar to the morphology of this virus. Similar to SAV, *Mengovirus* is a single-stranded positive-sense RNA virus with icosahedral symmetry (Strauss & Strauss 1994, Weaver & Frolov 2010, Jiang et al. 2014, Taksdal & Sindre 2016). However, the morphology of *Mengovirus* is different from SAV in that the former is non-enveloped, with a total genome length of ~ 8 kb and with a diametrical size that is half of SAV (i.e. 30 nm in diameter) (Luo et al. 1987, Strauss & Strauss 1994, Weaver & Frolov 2010, Carocci & Bakkali-Kassimi 2012, Jiang et al. 2014, Taksdal & Sindre 2016). Nevertheless, *Mengovirus* has previously been used as a process control for environmental samples (Nenonen et al. 2014, Haramoto et al. 2018, Sun et al. 2019) and follows the ISO criteria for determining *hepatitis A virus* and *norovirus* in food using RT-qPCR (ISO 2017). Therefore, it was considered sufficient in **Paper III** as a control of the methodology's performance by undergoing the same processing (e.g. filtration, elution, extraction and RT-qPCR) as for the viral target (i.e. SAV). This means, a sudden drop of the *Mengovirus* recovery could indicate a fault with the processing of the sample.

Table 5. *The water filtration methods used by different studies for detection of SAV.*

Type of study	SAV subtype	Filter	Buffer	Analysis	Type of water - Volume	Reference
Exp. challenge trial	SAV3	MDS+	Lysis[a]	RT-qPCR	SW - 1 L	(Andersen et al. 2010)
Exp. challenge trial	SAV1/ SAV3	cellulose ultrafiltration disc	n.d.	RT-qPCR	FW - 0.5 L	(Graham et al. 2011)
Exp. challenge trial	SAV3	MDS+	L-15[b]	RT-qPCR	SW - 1 L	(Jarungsriapisit et al. 2016a)
Exp. challenge trial	SAV3	MDS+	Lysis[c]	RT-qPCR	SW - 1 L	(Jarungsriapisit et al. 2016b)
In vitro study	SAV3	MDS+ or MF-	Lysis[d] L-15[e] NaOH[f]	RT-qPCR	SW - 1 L	**Paper I**
Exp. challenge trial	SAV3	MDS+ or MF-	Lysis[d] L-15[e] NaOH[f]	RT-qPCR	SW - 1 L	**Paper II**
Field study	SAV	MF-	Extraction[g] and Lysis[d]	RT-qPCR	SW - 1 L	**Paper III**

Note: n.d. means no data ; SW = Seawater; FW = Freshwater ; MDS+ = 1MDS electropositive filter; MF- = Electronegative nitrocellulose MF hydrophilic membrane filter; [a] Lysis buffer (E.Z.N.A total RNA kit from OmegaBioTek); [b] L-15 supplemented with 10% FBS; [c] Lysis buffer (iPrep™ PureLink® Total RNA Kit, Invitrogen, USA.); [d] NucliSENS® easyMAG® Lysis Buffer (bioMérieux, Marcy l'Etoile, France); [e] L-15 medium (pH 9.0) + 2% FBS and L-15 medium (pH 7.3–7.75); [f] 1 mM NaOH (pH 9.5) (Sigma-Aldrich); [g] NucliSens® Extraction Buffer 1 (easyMAG®, bioMérieux, Marcy l'Etoile, France); RT-qPCR = reverse-transcription quantitative real-time polymerase chain reaction.

5.3. Censored data

5.3.1. LOD & LOQ

There are different methods of estimating the LOD and LOQ, as given in the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) Q2 (R1) guideline (EMEA 1995). The filter-buffer combination giving the highest SAV3 recovery and efficiency was further studied. This was done in order to estimate LOD and LOQ for SAV3 in 1 L of natural seawater by RT-qPCR and RT-ddPCR analysis when using this concentration method.

In **Paper I**, a serial dilution of cell cultivated SAV3 was prepared and added to the natural seawater in order to make a standard curve. The LOD and LOQ estimated by RT-qPCR and RT-ddPCR were thereby given by the lowest amount of SAV3 in the spiked seawater giving a positive result, while the LOQ was calculated from the highest dilution giving results with a low relative standard deviation (RSD < 25%).

In **Paper II**, both LOD and LOQ were estimated based on two different standard curves made of a serial dilution of purified cell cultivated SAV3 and analysed by RT-qPCR. In addition, these values were based on tank water for SAV detection by RT-qPCR, when using the electronegative filter combined with lysis buffer and adjusted with the recovery of 25 % that was found for RT-qPCR analysis in **Paper I**. Both LOD and LOQ estimated in **Paper II** were also used in **Paper III**.

5.3.2. Non-detects

Non-detects are samples that contain data below detection limits, so-called "left-censored" data, and is a common but often neglected issue in environmental microbiology, and there are different ways of handling this for quantitative microbial risk assessment (QMRA) (Canales et al. 2018). When using concentration methods for analyzing virus from water, variable and poor recovery efficiencies are common challenges (Petterson et al. 2015). Hence, when conducting the reliability analysis, it is crucial to consider the issue of censored data, especially the one that is "left-censored" with regards to, e.g. virus recovery efficiency. By performing the necessary adjustments of the viral concentration data, they could more closely reflect the reality (Petterson et al. 2015).

When there is no detection of viral RNA in the sample, it can either be because it was not present or present at very low concentrations. Thus, if SAV is not detected at a site during a generation, it may be because the fish population was never infected or because it was slaughtered before the viral shedding gave detectable amounts of viral RNA. For the latter case, the time between SAV-detections is unknown, and this data is therefore called "right-censored" as they are data above a particular value, but with a true value that is unknown (Canales et al. 2018). Therefore, it is important to determine detection limits before handling data.

Substitution with $\frac{LOD}{\sqrt{2}}$ has been stated to be the method that results in the least error, including the lowest dose and infection risk biases, and it is also the recommended method for handling the data at different degrees of censoring (i.e. medium, high, and severe) (Canales et al. 2018). However, if the data distribution is unknown, then lognormal maximum likelihood estimation (MLE) is preferred for estimating the mean and standard deviation (Canales et al. 2018). Missing values lower than LOD can then be replaced with random draws from the fitted distribution (Canales et al. 2018). In **Paper III**, a survival regression was conducted, assuming that the errors were following the Weibull distribution. The data below LOD were handled by imputing as random draws from a lognormal distribution, where the mean and the standard deviation were derived from a censored data using the Kaplan-Meier method (Canales et al. 2018, Lee 2020). The number of copies was then ln transformed to approximate normality.

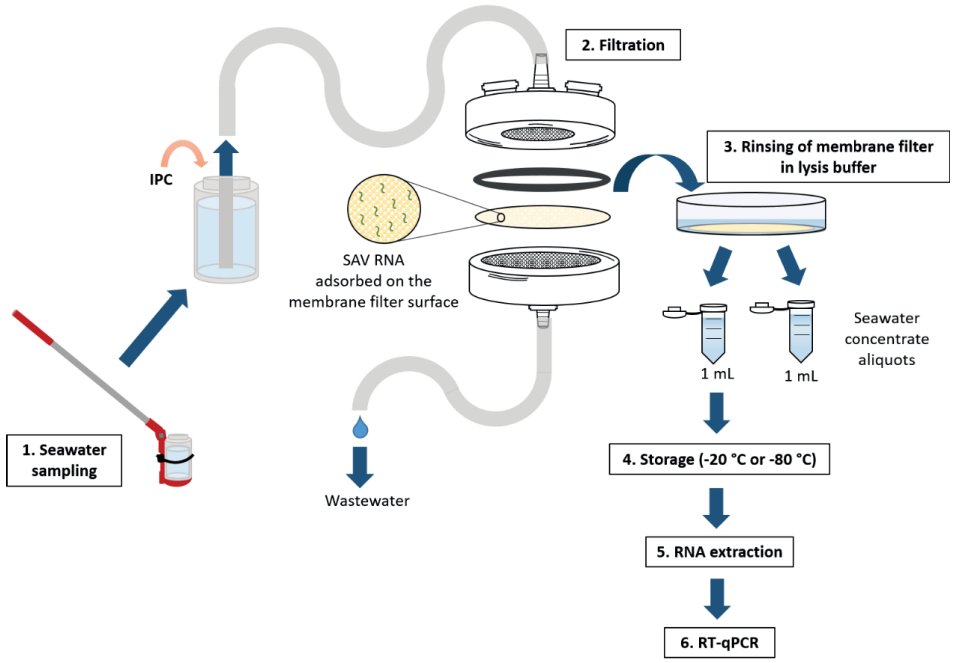

Figure 13. *Illustration showing the stepwise approach from sampling to analyzing 1 L seawater sample when using the filtration method for detecting SAV in seawater during the main field study (**Paper III**).*

6. Results

6.1. Concentration methods

Initially, five different combinations of two types of electrocharged filters (electronegative and electropositive) and four different eluents were developed, optimized and evaluated for the concentration of SAV3 in 1 L seawater (**Paper I & II**). It was revealed that MF- filter combined with the lysis buffer had the highest SAV3 recovery from natural seawater out of the five concentrations methods tested by using RT-ddPCR, followed by RT-qPCR (**Paper I**). It also appeared as if relatively more SAV3 was recovered from the higher dilutions of SAV3 compared to the lower dilutions when using this concentration method, suggesting that it may be more efficient at concentrating SAV3 at lower concentrations in seawater (**Paper I**).

The MF-/lysis buffer combination also returned significantly lower Cq values for SAV3 than all the other concentration methods in T_H, but was not significantly better in recovering SAV3 compared to the concentration method involving the combination between MDS+ with lysis buffer in T_L (**Paper II**). The MF-/lysis buffer combination was found the most successful in both the *in vitro* study (**Paper I**) and the challenge trial (**Paper II**). This concentration was therefore considered the most suitable method to apply under natural field conditions during which it also performed successfully (**Paper III**).

6.2. SAV-recovery

In **Paper I**, the SAV3 recovery was found to be 39.5 ± 1.8 % by RT-ddPCR and 25.9 ± 5.7 % by RT-qPCR, when using MF- filter combined with lysis buffer. More SAV3 was detected with RT-ddPCR compared to RT-qPCR; thus, RT-ddPCR appears to be more sensitive than RT-qPCR.

In **Paper II**, the SAV3 concentrations (based on Cq values) in tissue samples were overall higher than in tank water samples. This is an expected finding because fish (in contrast to water) serves as a replication site for the virus. The virus concentrations were generally higher in the heart samples compared to the mid-kidney (**Paper II**). However, a significant positive correlation could only be found between the levels of SAV3 in the tank water samples and in the mid-kidney samples collected from the cohabitant fish in both

T_L and T_H (**Paper II**). This observation moreover gives support to that the cohabitant fish provided a significant contribution to virus levels in the tank water throughout the challenge (**Paper II**).

The pairwise comparisons revealed that individually lysis buffer performed significantly better than 1 mM NaOH (pH 9.5) and L-15 medium (pH 9.0) and that the MF-filter performed significantly better than the MDS+ filter in T_H, but not in T_L (**Paper II**).

In **Paper III**, the MF-/lysis buffer revealed earlier detection in seawater than in fish samples, in all sites where the fish were tested positive for SAV. In addition, sampling from the top layer water within the net-pen did not only result in high levels of SAV but constituted by far the most practical method to be applied under field conditions during the pilot study, and therefore became the choice of the sampling procedure for the main field study (**Paper III**). In the main field study, it was found out that the lower SAV concentration in the seawater, the more days it took before SAV was detected in fish samples (**Paper III**).

6.3. LOD & LOQ

In the *in vitro* study, LOD and LOQ, when using the most suitable concentration method, were estimated as 200 and 5180 SAV3 RNA copies L^{-1} of natural seawater, as applicable to RT-ddPCR (**Paper I**). LOD was estimated as 42 SAV3 RNA copies L^{-1} of natural seawater, as applicable to RT-qPCR, while LOQ was not estimated by RT-qPCR because it would then only be based on one single biological water sample which is not enough to give an accurate value (**Paper I**). In the challenge trial, the LOD and LOQ were found to be 902 and 2736 SAV3 RNA copies L^{-1}, respectively, by RT-qPCR analysis, for tank water when using the most suitable concentration method (**Paper II**).

7. General discussion

This PhD study aimed at developing and optimizing an alternative method for surveillance of SAV at fish farms, by using a more cost-efficient, straightforward, resource-saving, time-saving, and animal welfare-friendly approach, based on the detection of SAV in seawater. Five different filter-buffer combinations were used to concentrate SAV from seawater. Initially, an *in vitro* study (**Paper I**) was performed to test out these five concentration methods, which were further investigated in a challenge trial (**Paper II**). In the present study, the filter-buffer combination (i.e. electronegative filter and lysis buffer) presenting the highest SAV recovery from seawater samples in **Paper I & II** was assessed under natural field conditions at several different Norwegian marine farm sites of Atlantic salmon, as presented in **Paper III**.

The water filtration method may allow earlier warning and implementation of disease control measures on the farm with identified SAV and in neighbouring farms compared to fish screening. Such a non-invasive surveillance method, based on seawater sampling, could serve as a warning system, which might significantly impact the health management of farmed Atlantic salmon.

7.1. Prevalence

Whether it is from fish tissue or water sample, the sensitivity of a detection method should not be defined by the amount of virus one is able to detect from the sample material. However, it should be based on the ability of the detection method to detect the virus at all when there is an actual prevalence of SAV infected fish in the population. Indeed, the virus concentration in water is greatly diluted, but the sensitivity of the detection method is the product of the probability of sampling an infected fish and the sensitivity of the individual test.

In order to compare the sensitivity of the water sampling method to a surveillance method based on the sampling of fish under field conditions, the comparison will be between the LOD for the water sampling method on the one side and the probability of sampling an infected fish on the other. For both methods, the sensitivity will obviously depend on the prevalence of SAV infected fish in the population. In the field, the probability of detection on which current sampling/monitoring approaches are used will

additionally depend on the farming conditions (i.e. the number of operative net-pens in the site and the distribution of prevalence between these net-pens).

In our study, we assumed that prevalence is evenly distributed between the net-pens, meaning the answer is independent of how many net-pens we have. For example, with a prevalence of 0.2 and 20 fish in sampling, the probability of detecting is 0.99, based on the formula $1-((1-P)^n)$, where P represents the chance of sampling an infected fish, out of n fish. This is highly related to the aspect of sacrifice because in the beginning of an outbreak with few infected fish individuals, the prevalence of SAV infected fish in the population will be low in the fish population, meaning the likelihood of identifying the infected fish is small. For these reasons, sampling more fish would be needed to increase the sensitivity of the sampling and the detection method combined. Thus, a water sample serves as a representation of the aquatic environment of a large number of fish at a farm site since the water sampling method can detect the virus at a low prevalence of SAV in the fish population.

Furthermore, the sites with positive water detection and no fish detection of SAV/PD in **Paper III** could be explained by that some fish in the site shed SAV, but that the prevalence was so small that the sampled fish thereby never was able to include any of the infected fish in the screening. Namely, it may be that if the fish had been monitored over a longer period at the site, then the prevalence would have increased, so that we would finally have a SAV positive result from the fish. However, it could also be the case that an escalating prevalence never happen, because the fish are healthy and not stressed. In this context, the role of the "epidemiological triad" (Snieszko 1974) becomes obvious, because in order to get an infection, there must be a pathogen, host and an environment that allows infection. It may therefore be that the environment does not allow an escalation of the situation.

In the main field study, we compared surveillance of SAV in marine farm sites of Atlantic salmon by sampling fish versus sampling seawater for the purpose of screening for SAV at the sites (**Paper III**). Results showed that detection of SAV was made earlier in seawater than in fish samples in all sites where the fish tested positive for SAV. Thus, the water filtration method may increase the probability for early detection of SAV in a fish farm, meaning this new method may serve as a possible alternative to the sacrifice of thousands of fish every year. This also means that the initiation of disease control

measures on farms neighbouring to farms with identified SAV could be implemented at an early stage so that further spread of the virus can be prevented.

In **Paper III**, the majority of the SAV-positive seawater samples were below LOD, which could indicate that the filtration method or the screening design need some further adjustments, such as filtering more samples each month, larger volumes (e.g. 5 L) or implementing more frequent collections of samples from all net-pens. However, the actual recovery of the virus will vary from sample to sample, meaning that the LOD, which was estimated in the challenge trial with tank water when analyzed by RT-qPCR (**Paper II**), is estimated too high for the seawater samples collected from the field (**Paper III**). In other words, LOD was a "conservative" estimate, which did not overestimate the sensitivity of the water filtration method.

7.2. Surveillance of pathogens in water

Water surveillance has shown potential and success for monitoring, e.g. enteric viruses (Borgmästars et al. 2017, Bisseux et al. 2018, Haramoto et al. 2018), poliovirus (WHO 2015) and COVID-19 (WRF 2020). Based on this, it strengthens the reason to believe that the water filtration has the potential to be applied for surveillance of SAV from seawater collected at fish farms.

Throughout the years, significant progress has been made in the development of methods for concentrating low concentrations of pathogens based on monitoring the water for environmental DNA (eDNA), as reported for the oomycete *A. astaci*, causing the crayfish plaque (Strand et al. 2011, 2014, Strand 2015, Rusch et al. 2020) and for the fish ectoparasite *G. salaris* (Rusch et al. 2018). For *A. astaci*, it has even become an established surveillance program, where DNA from spores of *A. astaci* are detected directly from water filtrates (Strand et al. 2020). The term environmental DNA/RNA (eDNA/eRNA) is coined to represent the detection of an organism from shed DNA/RNA, not the organism itself (Adams et al. 2019). However in this study, we are detecting the pathogen (SAV) from the seawater that represents the environment of the fish cohort in this study. Nevertheless, the above-mentioned eDNA-based studies, along with some experimental studies for SAV detection in seawater (Andersen et al. 2010, Jarungsriapisit et al. 2016a,b), provided some valuable advice, which were useful for the development and optimization of the water filtration method in this study.

7.3. Diagnostic tests

OIE has evaluated a few laboratory tests at the diagnostic level, of which RT-qPCR has been recommended as a screening tool (OIE 2019). A previous study by Jansen et al. (2019) evaluated the sensitivity and specificity for five different laboratory tests to verify the presence of SAV in fish tissue, including RT-qPCR, cell culture, virus neutralization test, histopathology, and IHC, that all are direct independent tests (Jansen et al. 2019). Also, that study found out that RT-qPCR (which was used in this study) has a high sensitivity and that it could be recommended as a screening test to determine a salmonid population's SAV infection status at marine farm sites (Jansen et al. 2019).

7.3.1. Validation

Validating a diagnostic assay means assessing its accuracy and precision, and thereby determining the fitness of the assay (Jacobson & Wright 2019). However, the outcome is only as good as the quality of the samples used (Jacobson & Wright 2019). According to OIE, the assay can be designated as validated for its original intended purpose, provided that the performance characteristics (i.e. analytical characteristics, diagnostic characteristics and reproducibility) are completed (Jacobson & Wright 2019). The validated assay's status is maintained by monitoring the method's performance under routine use conditions over time (Jacobson & Wright 2019). In order to confirm that the water filtration method developed in this study is suitable for its intended purpose, the method was tested in three different study setups, including an *in vitro* study (**Paper I**), an experimental study (**Paper II**) and a field study (**Paper III**).

Several factors can influence assay performances. Thus, optimization should take place first, followed by testing of the assay's performance characteristics for standardization, repeatability, analytical sensitivity, analytical specificity, thresholds (cut-off values), diagnostic sensitivity, diagnostic specificity, reproducibility and fitness for its intended purpose (Jacobson & Wright 2019). In other words, validity is measured by sensitivity and specificity and must be derived from reliable and reproducible data. Depending on the results from the validation work, the assay can either be considered fit for its purpose, or it may need additional optimization (Jacobson & Wright 2019).

For validation, known amounts of viral particles were spiked into seawater samples that were concentrated and recovered in this study (**Paper I**). The SAV3 inoculum used was based on tissue samples of Atlantic salmon from verified PD cases

(Taksdal et al. 2015). This inoculum was also used for achieving a natural exposure route to the cohabitant fish via i.p. injection of the shedder fish, with a low SAV3 dose, a high SAV3 dose, and a virus-free cell culture medium (mock inoculum) (**Paper II**).

The water filtration method's reliability was evaluated by including a positive control, a negative control, and an IPC in each run. The negative control samples and the positive control samples were extracted and analysed to monitor the nucleic acid extraction and the PCR processes, which could cause false-positive and false-negative results. This was also done in order to account for the variation that might be observed between test runs or between different laboratories, using the same method. In this study, all water samples were analysed at the same laboratory and they were always run in technical replicates to capture technical variation during analysis. The cut-off value for RT-qPCR analysis was consistently set at Cq 40 for all runs, and the thresholds were determined as LOD and LOQ (**Paper I & II**).

In **Paper I**, visual estimation was used when estimating LOQ, and the reason for this choice was the lack of replicate samples in the study. The impact of repeatability was noticeable in **Paper I**, where the RT-ddPCR and RT-qPCR gave highly divergent LOD data, probably due to few biological replicates. For the SAV3 recoveries in **Paper I**, they were estimated based on four biological replicates and could thus be considered a more reliable value. However, a number of 20 repeated measurements is what is commonly acceptable when calculating LOD, yet a number of 10 repeated measurements have been considered sufficient (Wenzl et al. 2016).

Compared to **Paper I**, LOD and LOQ were estimated differently in **Paper II** & **III** , as they were found from two different standard curves based on a serial dilution of SAV3 RNA, according to the ICH Q2 (R1) guideline (EMEA 1995). Indeed these standard curves are based on pure SAV RNA, thus, LOD and LOQ found in **Paper II** apply to pure SAV RNA from water samples. This was taken into consideration by diluting all water samples and running them both undiluted and diluted (1:4) which enabled us to check all water samples for inhibition. Our RNA from the water samples were pure. These values reveal the sensitivity of the RT-qPCR in our laboratory. Thus, it is recommended that other laboratories establish their own LOD and LOQ because these values depend on factors other than just the quality of the seawater. Indeed it would have been preferable to perform the calibration in **Paper II & III** by using the same seawater collection, treatment and testing protocols as will be used for actual test samples. However, in practice it means

that LOD and LOQ have to be found for every single water quality, which can be challenging.

Later in the study it was discovered that the RT-ddPCR might not have been optimized properly for its purpose, thereby underestimating the absolute quantity of SAV in the water samples, which might explain the low LOD value by RT-qPCR given in **Paper I**. Subsequently, since the SAV3 concentration in the tank water samples were estimated based on a sample of purified SAV3 RNA (inter plate calibrator) which was quantified by RT-ddPCR, the SAV concentrations should therefore be higher than given in **Paper II**. This issue has already been taken into consideration in **Paper III**.

It has been stated that the reproducibility should be evaluated under the conditions of intended use in order to validate an assay (Jacobson & Wright 2019). In this study, the water filtration method was tested under natural field conditions (**Paper III**), following the *in vitro* (**Paper I**) and the experimental study (**Paper II**). Although only one water sample was collected from each net-pen at a site every month in the field study (**Paper III**), the water samples were collected over a time period of up to nine months (May 2019-January 2020) from seven different marine farm sites, with different environmental conditions (**Paper III**). In addition, in the statistical analysis, the water samples collected from all net-pens at each site were considered pseudo-replicates by including the mean SAV copy number L^{-1} from each site sampled at the same time in the analyses (**Paper III**).

7.4. A potential for future use

In 2021, there were almost 600 marine operative sites for Atlantic salmon and rainbow trout (Directorate of Fisheries 2021). According to the surveillance program for SAV/PD, a minimum of 20 fish needs to be collected at least once a month from each of these sites until SAV is eventually detected, or until the population has been slaughtered (Lovdata 2017). Roughly estimated, this means almost 12,000 fish are withdrawn every month, using an approach that relies on analyzing a relatively small number of fish that are supposed to be representing the whole population on a site.

An estimated cost, based on the assumption that the sampled fish represents fish that would weigh approximately 5 kg by the end of the production cycle, and that the export price of fish is approximately 50 NOK/kg (Directorate of Fisheries 2019b), reveals that each month, fish with a value of 3 million NOK goes solely to the purpose of screening

the farmed fish, for the surveillance program of SAV/PD in Norway. The ongoing monitoring of SAV for surveillance in fish farms could be rationalized through a more cost-efficient, time-saving and less resource-demanding method, such as one that involves water sampling from a fish population's aquatic environment.

The present work has demonstrated a water filtration method with the potential to be applied as an alternative to the screening of fish for surveillance of SAV. This alternative method has successfully recovered SAV from water even at the early stages of SAV infection (**Paper II & III**). Therefore, this water filtration method may allow for earlier detection of SAV than the screening of relatively few fish individuals, in connection with the mandatory surveillance program for SAV/PD. It might have a significant impact on Atlantic salmon health management by serving as an early warning also for other harmful fish pathogenic viruses and may also be applied for other farmed fish species worldwide. Hence, this study has direct relevance for safeguarding fish health which could improve the sustainability and long-term profitability of the salmon industry in Norway and aquaculture internationally. However, further optimization and validation of this new method are required to allow the method's implementation for surveillance of SAV. Moreover, if seawater samples are identified as positive for any pathogen in a screening program, a diagnosis must still be confirmed by subsequent sampling and testing of fish. Nevertheless, by sampling water for surveillance of pathogens, the overall sacrifice of fish will be greatly reduced and the costs connected to it. Earlier detection can also initiate subsequent implementation of disease control measures on neighbouring farms, allowing improved biosecurity measures and more effective marine-farmed Atlantic salmon health management.

8. General conclusions

The *in vitro* study of SAV3 detection in artificial and natural seawater using either of five different concentration methods, followed by RT-qPCR and RT-ddPCR analysis (**Paper I**), resulted in the following main conclusions:

- SAV3 spiked in artificial, and natural seawater can be recovered after concentration by filtration of water samples.
- The MF- filter provided better virus recovery than the MDS+ filter, when lysis buffer was used to elute SAV3 RNA from the filter.
- More SAV3 was detected with RT-ddPCR compared to RT-qPCR; thus, RT-ddPCR appears to be more sensitive than RT-qPCR.
    - The SAV3 recovery was found to be 39.5 ± 1.8 % by RT-ddPCR and 25.9 ± 5.7 % by RT-qPCR, when using MF- filter combined with lysis buffer.
    - The LOQ and LOD were estimated as 5180 and 200 SAV3 RNA copies L^{-1} of natural seawater, by RT-ddPCR, while LOD was estimated as 42 SAV3 RNA copies L^{-1} of natural seawater by RT-qPCR.
- It appears that relatively more SAV3 was recovered from the higher dilutions of SAV3 compared to the lower dilutions when using the MF- filter combined with lysis buffer, suggesting that this method may be more efficient at concentrating SAV3 at lower concentrations in seawater.

The evaluation of the five different concentration methods followed by RT-qPCR analysis for SAV3 detection in seawater collected during a cohabitant challenge trial with Atlantic salmon post-smolts (**Paper II**), resulted in the following main conclusions:

- The concentration method combining the MF- filter and lysis buffer was found to result in an overall higher SAV3 recovery and the most consistent SAV3 detection in tank water samples, compared to the other concentration methods.
- The MF- filter and the lysis buffer generally performed better in seawater samples compared to the other buffer solutions and the MDS+ filter used herein.
- LOD and LOQ for SAV3, by RT-qPCR and when using the filtration method, were estimated as 902 and 2736 SAV3 RNA copies L^{-1}, respectively.

- A significant positive correlation was found throughout the challenge between the levels of SAV3 in the tank water samples and in the mid-kidney samples collected from the cohabitant fish.
- The combination of a MF- filter and lysis buffer is considered to have the best potential in serving as a more cost-efficient, straightforward, rapid, reliable, reproducible and animal welfare-friendly method for concentration and detection of SAV3 and potentially other SAV subtypes from seawater.

The assessment of the filtration method for SAV detection in seawater, collected from several different Norwegian marine farm sites of Atlantic salmon, in connection with the surveillance program for SAV/PD (**Paper III**), resulted in the following main conclusions:

- Earlier SAV detection was made in seawater than in fish samples, in all sites where the fish were tested positive for SAV.
- The lower the SAV concentration in the seawater, the more days it took before SAV was detected in fish samples.
- SAV detection in the seawater was done at sites with varying water temperatures, with the highest SAV concentrations found in the colder months.
- If seawater samples are identified as SAV-positive in connection with surveillance, then infection and a PD diagnosis in the fish population must still be confirmed by subsequent sampling and testing of fish.
- The filtration method has great potential as an alternative method and is a more straightforward, cost-efficient, time-saving, resource-saving, and animal welfare-friendly approach for detecting SAV in fish farms, compared to traditional methods for surveillance of this virus in fish farms. It may also have the potential for surveillance of other pathogens in farmed fish populations.

9. Future perspectives

Further optimization

The new filtration method successfully detected SAV from seawater even at the earlier stages of infection and thus shows potential to be applied for surveillance. However, further optimization and validation of this new method are required to allow method implementation for surveillance of SAV. This could be done by investigating this alternative method's performance at different levels of prevalence for further optimization for a longer duration. In relation to detection of SAV in the seawater when no fish is detected with SAV/PD in the screening, could be explained by that some fish in the site shed SAV, but that the prevalence is so small that the sampled fish does not contain any of the infected fish in the screening. This is something that can be investigated further in new studies, where we ask if there are risk factors for detecting a disease with traditional screening, after detecting SAV in water. Could it be, for example, that the prevalence begins to increase after lice treatments or other handling of the fish? Future studies should also consider filtering more water samples (and possibly larger water volumes) each month or implementing more frequent collections of water samples from all net-pens from several different sites that should be followed for at least one generation. Such studies could further reveal the water filtration method's potential and allow the method's implementation as an alternative method for surveillance of SAV in fish farms.

New applications

A future research objective will be to achieve the detection of several different infectious agents in a water sample. This expansion of the screening method could improve safeguarding in fish farms and result in gaining a higher sensitivity through more effective and early detections. Thus, the development of universal molecular methods for the detection of multiple infectious agents from a water sample would be of interest in the future. In this context, it would be interesting to look into the filtration mechanics and thereby learn more about what different factors are mainly impacting the adsorption to the filter by looking at different infectious agents. In the long run, it could also be beneficial to perform monitoring of the water continuously at the site by using an automatic sampling robot, which could facilitate and make regular water sampling more efficient.

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11. Scientific papers I-III

Paper I

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Development and evaluation of a method for concentration and detection of salmonid alphavirus from seawater

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ABSTRACT

Waterborne viral infections represent a major threat to fish health. For many viruses, understanding the interplay between pathogens, host and environment presents a major hurdle for transmission. *Salmonid alphavirus* (SAV) can infect and cause pancreas disease (PD) in farmed salmonids in seawater. During infection, SAV is excreted from infected fish to the seawater. We evaluated two types of filters and four different eluents, for concentration of SAV3. One L of seawater was spiked with SAV3, followed by filtration and virus elution from membrane filters. For the negatively charged MF hydrophilic membrane filter (MF-) combined with NucliSENS® lysis buffer the SAV3 recovery was 39.5 ± 1.8 % by RT-ddPCR and 25.9 ± 5.7 % by RT-qPCR. The recovery using the positively charged 1 MDS Zeta Plus® Virosorb® membrane filter (MD+), combined with NucliSENS® lysis buffer was 19.0 ± 0.1 % by RT-ddPCR and 13.3 ± 3.8 % by RT-qPCR. The limits of quantification (LOQ) and detection (LOD) were estimated to be 5.18×10^3 and 2.0×10^2 SAV3 copies/L of natural seawater, by RT-ddPCR. SAV3 recovery from small volumes of seawater, and the requirement for standard laboratory equipment, suggest the MF-filter combined with NucliSENS® lysis buffer would be a candidate for further validation in experimental trials.

1. Introduction

Salmonid alphavirus (SAV) is a positive-sense single-stranded RNA virus that belongs to the family *Togaviridae*, genus Alphavirus and encodes structural (E1-E3 and capsid) (Fringuelli et al., 2008), and non-structural proteins nsP1–nsP4 (Weston et al., 1999). The virus is divided into six subtypes (SAV 1–6), (Fringuelli et al., 2008; Hodneland et al., 2005; Karlsen et al., 2006; Weston et al., 1999), and is the causative agent of pancreas disease (PD) in farmed Atlantic salmon, *Salmo salar* L., and rainbow trout, *Oncorhynchus mykiss* (Walbaum) (Weston et al., 1999). Pancreas disease was first discovered in Scotland in 1976 (Munro et al., 1984), and since then several disease cases have been reported also in other countries (Kent and Elston, 1987; Munro et al., 1984; Murphy et al., 1992; Poppe et al., 1989; Raynard et al., 1992).

In Norway, PD has been diagnosed annually in farmed Atlantic salmon and rainbow trout since the mid-1980s (Olsen and Wangel, 1997). The disease is now considered endemic along the coast of Southwest-, West- and Mid-Norway, while the region of Northern Norway is considered a non-endemic area (Aunsmo et al., 2010; Hjeltnes, 2018). SAV subtype 3 (SAV3) was originally the only SAV subtype known to Norwegian aquaculture. However, since 2011, SAV2 has caused multiple outbreaks of disease in Mid-Norway (Hjeltnes et al., 2016; Hjortaas et al., 2016). The disease occurs mostly during the seawater production phase. Current knowledge indicates that transmission of SAV occurs between neighbouring seawater farms mainly through water currents, while long distance spread is a result of transport of live fish carrying the infection (e.g., smolt, and fish for slaughter) (Haredasht et al., 2019; McLoughlin et al., 2006, 2003; McVicar, 1987; Rodger and Mitchell, 2007; Stene et al., 2014). In Norway, PD has been classified as a notifiable disease since 2007, and control measures have been implemented in order to prevent further spread to areas free of the disease, as well as to reduce losses within the endemic zones. According to revised legislation from 2017, the control of PD requires monthly sampling of fish from all marine farming sites holding salmonid fish for testing by polymerase chain reaction (PCR). This surveillance method leads to sacrificing thousands of fish every year, and is problematic both in relation to aquaculture economy, as well as to animal welfare. For this reason, detection of the virus excreted into the seawater surrounding the fish populations would be more cost effective than sampling of fish, and potentially yield data on virus presence earlier in the course of the

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infection.

Collection of water samples, followed by concentration of dispersed biological material, has been recommended as a method for environmental surveillance, so-called e-DNA (Organization and Initiative, 2015). The method has previously been used on tap water, groundwater, fresh- and seawater to concentrate different pathogens (Abbaszadegan et al., 1993; Logan et al., 1980, 1981; Nupen and Bateman, 1985; Singh and Gerba, 1983; Sobsey et al., 1985). Since salts and other inhibitors in environmental seawater may have influence on virus detection by quantitative RT-PCR (RT-qPCR) (Rački et al., 2014), RT-ddPCR (RT-droplet digital PCR) technology might be favourable. RT-ddPCR has reduced sensitivity to inhibitors and provides direct quantification of the target (Burns et al., 2010; Lui and Tan, 2014; Rački et al., 2014).

In the present study, a first step towards the development of a new surveillance method for SAV in salmonid populations in seawater was initiated. Two different membrane filters and four different elution buffers were evaluated for the concentration of SAV3 from seawater. The virus was spiked into artificial and natural seawater, adsorbed to and eluted from charged filters before quantification with RT-qPCR and RT-ddPCR.

2. Material and methods

2.1. Virus and cell culture

The SAV3 isolate used for this study was originally isolated from pooled heart and head kidney samples of Atlantic salmon from the Hordaland region of Norway (Taksdal et al., 2015). The CHSE-214 cell line (ATCC CRL-1681) derived from a Chinook salmon (*Oncorhynchus tshawytscha*) embryo was used in the propagation of SAV3. Briefly, cells were grown at 20 °C in T-150 culture flasks containing Leibowitz L-15 medium (Life Technologies, UK) supplemented with 10 % foetal calf serum (FBS) and gentamicin (Lonza, USA). The SAV3 isolate was used to inoculate the cell culture, and after seven-days incubation at 15 °C, the culture supernatants were harvested, centrifuged, and 1 mL aliquots were stored at −80 °C before been used for spiking. SAV3 copies in 1 mL of stock (1.62×10^7 copies) used for spiking 1 L of artificial seawater and natural seawater, was quantified by RT-ddPCR.

2.2. Seawater samples

Artificial seawater (Sigma-Aldrich, Germany) in one litre (1 L) polyethylene bottles was stored at 4 °C in the dark before use. Natural seawater in 1 L bottles was obtained from the Oslofjord (kindly provided by the Norwegian Institute for Water Research (NIVA), Solbergstrand research station).

2.3. Filters and buffers

The negatively charged nitrocellulose MF hydrophilic membrane filter (MF-, 47 mm diameter and 0.45-μm pore size) (Millipore, USA) and positively charged 1 MDS Zeta Plus® Virosorb® membrane filter (MD+, 47 mm diameter) (Cuno, Meriden, Conn, USA) were used. In order to develop a new concentration method, preliminary tests were performed to determine the efficiency of MF- and MD + filters for adsorption of SAV3, using the following buffers for elution: (1) NucliSENS® lysis buffer (bioMérieux SA, France); (2) 1 mM NaOH (pH 9.5) buffer; (3) L-15 + 2% FBS (pH 9.0) buffer and (4) L-15 + 2% FBS buffer.

2.4. Initial testing of seawater filtration

The following protocol was used for both MF- and MD + filters. One litre of either artificial seawater or natural seawater were spiked with 1 mL of SAV3 stock (1.62×10^7 copies). The filters were placed into a filter holder, which was fitted to a Masterflex® E/S™ portable environmental sampler (Cole-Parmer Instrument Company, USA), and the water was pumped at a flow rate of 200 mL/min as shown in Fig. 1. After filtration of the spiked seawater, the filter was immediately placed into a Petri dish containing 2.4 mL of buffer 1 and shaken (30 min, 600 RPM, room temperature) to release the membrane-bound virus. For elution with buffer 2, 3 and 4, each filter was cut and placed in a 50 mL Falcon tube containing 4 mL of the respective buffer. Each sample was vortexed (3×1 min, with a 5 min interval at room temperature) and the eluates (concentrates) were stored at −80 °C prior to RNA extraction and SAV3 detection by RT-qPCR and RT-ddPCR. For both types of seawater, a negative control sample without SAV3 spiking was analysed to verify the absence of any natural SAV contaminants. The recovery percentage was calculated based on the formula:

$$\text{SAV3 recovery } (\%) : \frac{SAV3\ particles\ in\ seawater\ concentrate}{SAV3\ particles\ in\ spiked\ seawater} x\ 100$$

As the initial study, using two biological replicates, showed that the MF-/buffer 1 combination gave the highest virus recovery, this combination was further studied in order to assess the efficiency of the method and the limit of detection (LOD) and limit of quantification (LOQ) for SAV3 in 1 L of natural seawater.

2.5. MF-filter concentration method in 1 L of seawater

To further assess the MF-/buffer 1 method for concentration of SAV3 in seawater, a two-fold serial dilution (1:1 to 1: 512) was prepared from the SAV3 stock. Each stock dilution was used to spike 1 L of natural seawater, followed by concentration and elution as described in 2.4. The study was performed four times. RNA was isolated from the spiked seawater concentrate (eluate). SAV3 RNA was quantified using RT-ddPCR and RT-qPCR, and the recovery percentage was calculated.

2.6. Limit of quantification (LOQ) and limit of detection (LOD) of SAV3 in 1 L of natural seawater

In order to estimate the LOQ and LOD for SAV3, a five-fold dilution series (1: 1 to 1: 5^{11}) of the SAV3 stock was prepared. The virus dilutions were used to spike 1 L samples of natural seawater, and the setup was performed twice. The seawater was processed by the MF-/buffer 1 method and RNA extracted from the eluates as described in 2.4. The LOD as estimated by RT-qPCR and RT-ddPCR was given by the lowest amount of SAV3 giving a positive result in the seawater sample, while the RT-ddPCR LOQ was calculated from the highest dilution giving results with a low SD. The SAV3 recovery in the dilution series was also assessed to further evaluate the MF-/buffer 1 method for concentration.

2.7. RNA extraction and RT-qPCR

Total RNA was extracted from 1 mL of SAV3 concentrate from 1 L spiked artificial and natural seawater, according to the generic easyMAG protocol (bioMèrieux, Marcy l'Etoile, France). The RNA was eluted in 50 μL buffer and stored at −80 °C prior to RT-qPCR, using the TaqMan® Fast Virus 1-Step Master Mix kit (Applied Biosystems, USA). The SAV Q-nsP1 primers and probe (F-primer: 5′−CCGGCCCTGAACCAGTT- 3′, R-primer: 5′-GTAGCCAAGTGGGAGAAAGCT-3′ and probe: 5′ FAM-CTGGCCACCACTTCGA-3′ -MGB') were used, which generated a 107-bp PCR-product (Hodneland and Endresen, 2006). A total volume of 20 μL RT-qPCR mix contained 500 nM of each primer, 300 nM probe and 2 μL of RNA. The RT-qPCR reaction was run in duplicates in an Agilent AriaMx PCR System cycler from Applied Biosystems, using the following conditions: reverse transcription at 50 °C for 5 min, initial denaturation at 95 °C for 2 min, and 45 cycles of amplification (94 °C for 15 s, 60 °C for 40 s). Potential inhibition of SAV3 RT-qPCR from the natural seawater was initially evaluated by analysing 1:1 and 1:4 dilutions of the RNA. For samples showing inhibition, the 1:4 dilution was used to estimate virus recovery. Absolute quantification of SAV3 was performed using the formula: $N1 = N2 * (1 + E)^{(CqN2 - CqN1)}$, where N1 and N2 denote

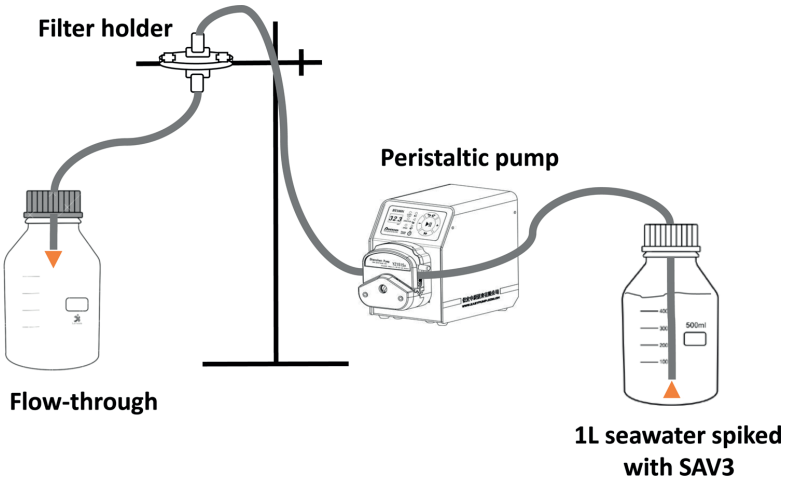

Fig. 1. Schematic diagram of seawater concentration setup.

virus copy numbers in the samples and spike, respectively, *E* is the amplification efficiency of the SAV3 RT-qPCR (104 % = 1.04) and Cq is the cycle quantification.

2.8. Reverse transcription droplet digital PCR (RT-ddPCR)

The primers and probe used for the RT-qPCR were also used for the RT-ddPCR assay. The RT-ddPCR was performed in 20 µL volumes using the One-step RT-ddPCR Advanced Kit for Probe (Bio-Rad), as described by the manufacturer, with 1.8 µL of RNA, and primers and probe at final concentrations of 900 nM and 250 nM, respectively. Droplets were generated on a QX-200 droplet generator (Bio-Rad), before PCR was performed on a T100 instrument, using the following thermal conditions: 60℃ for 60 min, 95℃ for 10 min, followed by 45 cycles of 95℃ for 30 s, 60℃ for 1 min, and a final 98℃ for 10 min. The RT-ddPCR plate was analysed in a QX200™ Droplet Reader (Bio-Rad, USA) using QuantaSoft™ software (Bio-Rad, USA), which was also used for data analysis. The threshold for distinguishing positive from negative droplets was determined manually, as the intensity in relative fluorescence units (RFU) above which no droplet signal would be expected in the non-template controls (NTC). Samples were considered positive for the marker if they contained three or more positive droplets.

2.9. Statistical analysis

All statistical analyses were performed using graph-pad prism 8.3.0. First data was checked for normality, and they were normally distributed. A two-way ANOVA was used to compare differences in SAV3 recovery between the RT-ddPCR and RT-qPCR assays from two-fold serial dilutions (1:1 to 1: 512). A difference of $p < 0.05$ was considered statistically significant. Fishers Least Significant Difference test was used to compare dilutions within the group and between the two groups, and no correction for multiple comparison was made.

3. Results

3.1. Evaluation of filters and buffers for virus recovery

Two different types of membrane filters (one positively and one negatively charged) and four different buffers (buffer 1–4) were evaluated for their capacity to concentrate and elute SAV3 from 1 L seawater samples. The MF- filter/buffer 1 and MD + filter/buffer 1 methods produced the best SAV3 recoveries from natural seawater with 39.5 ± 1.8 % and 19.0 ± 0.1 % (mean ± standard deviation), when the samples were analysed by RT-ddPCR (Table 1). The virus recovery was higher from natural seawater, compared to artificial seawater, when either the MF- filter/buffer 1 or the MD + filter/buffer 1 method was applied. Overall, the highest SAV3 recovery of 39.5 ± 1.8 % was achieved from natural seawater by RT-ddPCR, using the MF- filter/buffer 1

Table 1
Recovery rate (%) of SAV3 in spiked artificial and natural seawater. One litre of seawater was spiked with 1.62×10^7 copies of SAV3 and concentrated, using a negatively charged (MF-) or a positively charged (MD+) filter. Different buffers were used for elution, and SAV3 was quantified with RT-qPCR and RT-ddPCR. SAV3 recovery is presented as mean ± standard deviation from two biological replicates. ND = Not detected.

Membrane	Sample type	Elution buffer	SAV3 recovery (%)	
			RT-qPCR	RT-ddPCR
MF negatively charged filter	Artificial seawater	NucliSENS® lysis buffer	23.7 ± 6.7	38.2 ± 2.9
		1m M NaOH pH 9.0	0.4 ± 0.2	0.4 ± 0.1
		L-15 + 2% FBS pH 9.0	0.4 ± 0.1	0.3 ± 0.1
		L-15 + 2% FBS	0.2 ± 0.1	0.2 ± 0.1
	Natural seawater	NucliSENS® lysis buffer	25.9 ± 5.7	39.5 ± 1.8
		1m M NaOH pH 9.0	1.3 ± 0.4	3.7 ± 2.6
		L-15 + 2% FBS pH 9.0	0.8 ± 0.4	1.7 ± 1.4
		L-15 + 2% FBS	0.7 ± 0.2	1.6 ± 0.3
MD positively charged filter	Artificial seawater	NucliSENS® lysis buffer	6.2 ± 2.5	7.8 ± 4.2
		1m M NaOH pH 9.0	ND	ND
		L-15 + 2% FBS pH 9.0	ND	ND
		L-15 + 2% FBS	ND	ND
	Natural seawater	NucliSENS® lysis buffer	13.3 ± 3.8	19.0 ± 0.1
		1m M NaOH pH 9.0	ND	ND
		L-15 + 2% FBS pH 9.0	ND	ND
		L-15 + 2% FBS	ND	0.3 ± 0.4

method. Furthermore, all tests using buffers 2, 3 and 4 resulted in no or very low recoveries (Table 1).

3.2. The MF-filter/buffer 1 concentration method

Given that our initial experiments showed the MF-filter/buffer 1 method gave the best virus recovery, when 1.62×10^7 SAV3 copies were used to spike 1 L of natural seawater (Table 1), the MF-filter was further evaluated. A two-fold serial dilution (1:1 to 1: 512) was prepared from the SAV3 stock and used to assess the MF-filter capacity for concentration of SAV3 in natural seawater. The results showed the SAV3 recoveries with the MF-filter/buffer 1 method from 1:1 dilution of the dilution series (23.1 ± 5.3 % and 43.1 ± 7.1 %) were similar to the recoveries from our initial experiment (25.9 ± 5.7 % and 39.5 ± 1.8 %) in both RT-qPCR and RT-ddPCR, respectively. Furthermore, results from both RT-ddPCR and RT-qPCR assays of the 2-fold serial dilution (1:2 to 1: 512) of the SAV3 spiked seawater, revealed relatively more SAV3 was recovered from higher dilutions (Table 2). Comparison of results from dilution per group (1:1 to 1: 512), revealed statistically significant increase in SAV3 recovery by RT-qPCR *($p < 0.04$ to 0.0001)*. Similarly, results from dilution per group (1:1 to 1: 512) for RT-ddPCR, also showed statistical increase in SAV3 recovery *($p < 0.04$ to $p < 0.001$)* (Table 2).

3.3. LOD and LOQ of SAV3 in 1 L natural seawater concentrated by the MF-/buffer 1 method

Eleven five-fold dilutions of SAV3 were prepared and added to the natural seawater. The RNA extracted from the natural seawater concentrate was analysed by RT-qPCR and RT-ddPCR to the lowest amount of SAV3 particles detected. As shown in Table 3, SAV3 was detected by both methods from the tested dilutions, except for $1{:}5^8$ to $1{:}5^{11}$ dilutions. The LOQ and LOD of RT-ddPCR were estimated to be 5.18×10^3 and 2.0×10^2 SAV3 copies/L of natural seawater respectively (n = 2), while the LOD for RT-qPCR was estimated to be 42 SAV3 copies/L of natural seawater (n = 1); where n is the number of biological replicates.

4. Discussion

The present study demonstrates that *Salmonid alphavirus* spiked in artificial and natural seawater can be recovered after concentration by filtration of water samples. The results showed that the negatively charged MF- filter in general provided better virus recovery than the positively charged MD + filter, and when NucliSENS® lysis buffer (buffer 1) was used to elute SAV RNA from the filter. However, the use of MD + filter in combination with buffer 1 also resulted in fairly good virus recoveries. Thus, the common denominator is that the buffer 1 has the decisive impact in the higher virus recovery, rather than the type of filter used for concentration. Forty percent SAV3 recovery from natural seawater with the MF-/buffer 1 method using RT-ddPCR, indicates a potential for the method to be used for SAV3 recovery under field conditions with farmed salmonids. The method can potentially co-concentrate other viruses well, hence the MF-/buffer 1 method of concentration and elution steps described here may enable accurate assessment of the viruses in seawater samples.

Table 2
Recovery rate (%) from a 2-fold serial dilution of SAV3 spiked into seawater. One litre of seawater was spiked with 2-fold dilution series of 1.62×10^7 copies of SAV3 and concentrated using MF-/buffer 1 concentration method. SAV3 recovery using RT-qPCR and RT-ddPCR is presented as mean ± standard deviation from four biological replicates. Two-way ANOVA was used to compare differences in SAV3 recovery between RT-ddPCR and RT-qPCR assays from two-fold serial dilutions.

SAV3 dilutions (copy number)	SAV3 recovery (%)	
	RT-qPCR	RT-ddPCR
1:1 (1.62×10^7)	23.1 ± 5.3	43.1 ± 7.1
1:2 (8.10×10^6)	37.3 ± 4.2	55.8 ± 1.6
1:4 (4.05×10^6)	41.3 ± 6.3	60.6 ± 3.3
1:8 (2.03×10^6)	40.7 ± 4.8*	66.0 ± 5.1*
1:16 (1.01×10^6)	39.6 ± 5.7**	68.6 ± 8.3**
1:32 (5.06×10^5)	57.2 ± 18.0	63.5 ± 9.1
1:64 (2.53×10^5)	56.1 ± 14.2	70.2 ± 4.7
1:128 (1.27×10^5)	46.6 ± 20.6	65.6 ± 2.9
1:256 (6.33×10^4)	48.8 ± 12.5***	78.0 ± 15.2***
1:512 (3.16×10^4)	62.2 ± 15.9	64.0 ± 22.7

Note: *$p < 0.04$, **$p < 0.01$ and ***$p < 0.02$ signs represent the levels of significance.

Table 3
Estimation of the lower limit of detection (LOD), the limit of quantification (LOQ) and the recovery rate (%) of SAV3 in 1 L spiked seawater. One litre of seawater was spiked with 5-fold dilution series (1:1 to 1: 5^{11}) of 1.62×10^7 copies of SAV3 and concentrated using the MF-/buffer 1 concentration method. The LOD for RT-qPCR and RT-ddPCR was given by the lowest amount of SAV3 giving a positive result in the seawater sample, while the RT-ddPCR LOQ was calculated from the highest dilution giving results with a low SD. SAV3 recovery for RT-ddPCR is presented as mean ± standard deviation from two biological replicates. For RT-qPCR, the recovery from one dilution series is given.

SAV3 dilutions (copy number)	SAV3 recovery (%)	
	RT-qPCR	RT-ddPCR
1:1 (1.62×10^7)	30.8	34.4 ± 9.6
1:5 (3.24×10^6)	38.9	37.3 ± 11.0
$1{:}5^2$ (6.48×10^5)	38.5	40.3 ± 9.5
$1{:}5^3$ (1.30×10^5)	34.1	36.2 ± 10.0
$1{:}5^4$ (2.59×10^4)	28.8	37.5 ± 6.2
$1{:}5^5$ (5.18×10^3)†	42.4	39.9 ± 4.5
$1{:}5^6$ (1.04×10^3)	28.9	47.1 ± 32.2
$1{:}5^7$ (207)‡	43.3	124.4 ± 120.1
$1{:}5^8$ (42)*	32.1	ND
$1{:}5^9$ (8)	ND	ND
$1{:}5^{10}$ (1.6)	ND	ND
$1{:}5^{11}$ (0)	ND	ND

Note: † represents (LOQ) for RT-ddPCR, ‡ represents LOD for RT-ddPCR while * represents LOD for RT-qPCR. ND = Not detected.

The MF- filter has been used in quantifying poliovirus from 1 L seawater (Katayama et al., 2002). The method required an acidification step to remove cations and promote elution (Fong and Lipp, 2005; Lukasik et al., 2000; SOBSEY, 1995; Sobsey et al., 1973). In the present study, the method used by Katayama and others was modified by directly lysing the adsorbed SAV3 with buffer 1. This modification was introduced in the method development for this study in order to circumvent the laborious acidification step, and to maximize virus recovery during the concentration process.

Previous studies have found that preconditioning of seawater samples with salts facilitates electrostatic adsorption of virus to MF-negative filters (Katayama et al., 2002). Enteric viruses, like polio virus, are negatively charged in water and thus requires cations in the water in order to be linked to a negatively charged membrane.(Katayama et al., 2002; Pallin et al., 1997). The protein capsid of viruses typically contains ionisable amino acids, such as glutamic acid, aspartic acid, histidine, and tyrosine (Gerba, 1984). Like many organic chemicals, these individual carboxyl and amino groups, depending on the pH of the surrounding environment, can gain or lose a proton, giving the capsid a net electrical charge. In this study, preconditioning of the filters was not performed, and it is unclear what kind of interaction was involved in the adsorption of SAV3. However, given that SAV3 is positively charged in seawater, and pH in the water is below the SAV isoelectric point (PI, 9.95) (Pickett et al., 2012), it is plausible for SAV to adsorb to a negatively charged filter, without the need for preconditioning.

The positively charged MD + filter with buffer 1 also facilitates adsorption of SAV from seawater. In this case, the presence of cations and anions in the seawater may have strengthened hydrophobic

reactions between SAV3 and filter, which led to SAV3 adsorption. Similar findings have been reported previously (Lukasik et al., 2000).

In addition, negatively charged filters have been used for recovery of solid-associated viruses from effluent, raw sewage and sludge samples (Agency, 1984; UKWIR, 2000; Wyn-Jones and Sellwood, 2001). In the current study, both artificial and natural seawater were used, and more virus was concentrated from natural seawater that contained organic matters compared to the artificial seawater, which correlates with previous reports (Agency, 1984; UKWIR, 2000; Wyn-Jones and Sellwood, 2001).

Inhibitions may cause major problems when detecting or quantifying RNA virus in seawater concentrates by RT-qPCR methods (Gibson et al., 2012; Girones et al., 2010). In an effort to increase detection sensitivity, the use of robust extraction and amplification methods is recommended, as organic matter that may interfere with the enzymes used for amplification are co-concentrated with viral particles. In the present study, RT-ddPCR was applied in order to overcome the problem of inhibition (Rački et al., 2014). We assessed the MF-/buffer 1 method capacity for concentration of SAV3 in seawater from 2-fold serial dilutions. It appears that relatively more SAV3 was recovered from the higher dilutions compared to the lower dilutions (Table 2), which may suggest that MF-filter may be more efficient at concentrating at higher dilutions of SAV3. This is interesting, as virus content in seawater is usually low, and a method that enhances recovery from environmental samples would be important for risk assessment and surveillance. Furthermore, we compared SAV3 recovery as quantified by RT-qPCR to RT-ddPCR. At lower dilution (1:1 to 1:4), there was no significant differences in SAV3 recoveries between RT-qPCR and RT-ddPCR (Table 2). However, at higher dilutions (from 1:8 to 1:16; $p < 0.04$, *0.01* and at 1: 256; $p < 0.01$), significant differences between SAV3 recovery from RT-ddPCR and RT-qPCR was observed. The RT-ddPCR and RT-PCR gave highly divergent LOD data, which is probably due to few biological replicates. More SAV3 was detected with RT-ddPCR compared to RT-qPCR. Thus, RT-ddPCR appears to be more sensitive than RT-qPCR in accordance with previous reports (Burns et al., 2010; Morisset et al., 2013). RT-ddPCR therefore is a useful alternative method for detection and quantification of SAV3 in seawater using the method with the negatively charged filter with NucliSENS® lysis buffer, and the assay may be extended to quantification of other seawater fish viruses.

In summary, the method developed in this study is capable of detecting *Salmonid alphavirus* subtype 3 from natural seawater samples. We hypothesize that the concentration method developed in this study has the potential to be applied for disease surveillance purposes in farmed populations of salmonid fish in seawater, and may be applied to detect other fish pathogenic viruses in water (e.g., *Infectious salmon anaemia virus*, *Salmon gill poxvirus*, *Piscine orthoreovirus*).

CRediT authorship contribution statement

Simon Chioma Weli: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition. **Lisa-Victoria Bernhardt:** Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - review & editing, Visualization. **Lars Qviller:** Writing - review & editing, Supervision. **Mette Myrmel:** Validation, Formal analysis, Data curation, Writing - review & editing, Visualization, Supervision. **Atle Lillehaug:** Conceptualization, Resources, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors have no competing interests.

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Paper II

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Concentration and detection of salmonid alphavirus in seawater during a post-smolt salmon (*Salmo salar*) cohabitant challenge

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ABSTRACT: Currently, the prevalence of salmonid alphavirus (SAV) in Norwegian Atlantic salmon farms is largely surveyed via sacrificing fish and sampling of organ tissue on a monthly basis. However, a more cost-efficient, straightforward, rapid, reliable, reproducible and animal welfare friendly method based on the detection of SAV in water could be considered as an alternative method. In the present study, such a method was developed and optimized through a 6 wk cohabitant challenge trial, using post-smolt Atlantic salmon *Salmo salar* L challenged with high or low doses of SAV subtype 3 (SAV3). Tank water and tissue samples from cohabitant fish were collected at 16 time points. SAV3 was concentrated from the water by filtration, using either electronegative or electropositive membrane filters, which were subsequently rinsed with one of 4 different buffer solutions. SAV3 was detected first in tank water (7 d post-challenge, DPC), and later in cohabitant fish organ tissue samples (12 DPC). The electronegative filter (MF-Millipore™) and rinsing with NucliSENS® easyMAG® Lysis Buffer presented the best SAV3 recovery. A significant positive correlation was found between SAV3 in the tank water concentrates and the midkidney samples. Based on these results, detection of SAV3 in filtrated seawater is believed to have the potential to serve as an alternative method for surveillance of SAV in Atlantic salmon farms.

KEY WORDS: SAV detection · SAV concentration · Water filtration · Surveillance · RT-qPCR · Membrane filters · Salmonid alphavirus · Pancreas disease

1. INTRODUCTION

Pancreas disease (PD) is considered to be one of the most serious virus diseases in sea-farmed salmonids (Sommerset et al. 2020), resulting in significant economic consequences (Jansen et al. 2015). PD was discovered for the first time in 1976 in Scotland (Munro et al. 1984) and first reported in Norway more than a decade later (Poppe et al. 1989). The aetiological agent for PD was first reported in 1995 (Nelson et al. 1995) and is described as an enveloped, positive-sense single-stranded RNA virus (+ssRNA) (Deperasińska et al. 2018), formally named *Salmonid alphavirus* (SAV) (Weston et al. 2002).

SAV belongs to the genus *Alphavirus* in the family *Togaviridae* (Nelson et al. 1995, Weston et al. 2002) and, based on phylogenetic analysis of the partial E2 gene and nsP3 gene in SAV from farmed Atlantic salmon *Salmo salar* L. and rainbow trout *Oncorhynchus mykiss*, this virus can be divided into 6 subtypes (i.e. SAV1–SAV6) (Fringuelli et al. 2008). In Norway, there are currently 2 known subtypes (SAV2 and SAV3), and they are primarily distributed into 2 different PD endemic zones (Hjortaas et al. 2016). SAV3

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has so far only been found in Norway, in aquaculture of rainbow trout and Atlantic salmon (Hodneland et al. 2005).

Various experimental challenge studies have shown that SAV spreads via horizontal transmission (McLoughlin et al. 1996, Andersen et al. 2010, Xu et al. 2012, Graham et al. 2012, Jarungsriapisit et al. 2016a,b, 2020). In some of these studies it was also possible to detect SAV directly from seawater (Andersen et al. 2010, Jarungsriapisit et al. 2016a,b, 2020). However, the current methods for detection of SAV and diagnosis of PD involve sampling of fish, followed by histopathology and quantitative real-time PCR (RT-qPCR). In 2007, PD became a notifiable fish disease (list 3) in Norway. National regulation was introduced in order to reduce the consequences of PD in a defined PD zone and to prevent further spread of SAV. Revision of the regulations in 2017 introduced different control zones for SAV2 and SAV3, as well as a surveillance zone.

The surveillance programme requires monthly sampling for PCR analysis of Atlantic salmon, trout, rainbow trout and char from all marine operative fish farming sites without current status as SAV-infected (Lovdata 2017). This results in the sacrifice of thousands of fish every year and is a time-consuming and resource-demanding approach that relies on analysing a relatively small number of fish that should represent the whole population on a site. Hence, in the beginning of an outbreak, when few individuals are infected, the likelihood of identifying the infected fish is small. A water sample that represents the environment for the total population in the farm may therefore serve a possible alternative due to fish shedding SAV into the water. Monitoring of the aquatic environment for harmful pathogens by filtration of water has been found to be successful, as reported for the oomycete *Aphanomyces astaci* which causes the crayfish plague (Strand et al. 2019, Rusch et al. 2020) and for the fish ectoparasite *Gyrodactylus salaris* (Rusch et al. 2018), and constitutes an animal welfare friendly method, with a potential for improved management strategies.

It has been reported that filtering water through charged membrane filters is a useful tool for concentrating viruses from water (Cashdollar & Wymer 2013). This simple and efficient technique is known as virus adsorption–elution (viradel), and has been described in several studies (Wallis et al. 1972, Farrah et al. 1976, Goyal & Gerba 1983), including SAV challenge trials (Andersen et al. 2010, Jarungsriapisit et al. 2016a,b, 2020). Recently, we carried out an *in vitro* study involving filtration of artificial and natural seawater that was spiked with SAV3 (Weli et al. 2021). Virus in water samples was concentrated by adsorption to either electronegative or electropositive membrane filters, followed by rinsing of filters with one of 4 different buffer solutions, and quantifying using RT-qPCR and reverse-transcription droplet digital PCR (RT-ddPCR).

In the present study, our aim was to evaluate the potential of these 5 methods in detecting SAV3 directly from seawater holding infected post-smolt Atlantic salmon. Therefore, a 6 wk cohabitant challenge study was performed, with sampling of tank water and fish tissue at 16 time points. An efficient concentration method (CM) for SAV in water may serve as a cost-efficient, straightforward, rapid, reliable and reproducible process for detection of SAV in Atlantic salmon farms compared to screening of fish. Such a method may result in earlier identification of infection in a fish population and prevention of further virus spread. This method may also be developed for other waterborne pathogenic viruses, thus enabling earlier disease control measures and improved virological safety of the water environment.

2. MATERIALS AND METHODS

2.1. Experimental design

A cohabitant challenge trial was carried out in 3 separate tanks for a duration of 38 d. The number of shedder fish and cohabitant fish in each tank was 15 and 55, respectively. During the challenge period, sampling of both tank water and cohabitant fish was performed at 16 time points (see Fig. 1).

2.2. Experimental fish

The Atlantic salmon used in this trial arrived as eyed eggs when they were approximately 370 degree-days and were reared at the fish facility at the Industrial and Aquatic Laboratory (ILAB, Bergen High Technology Centre). In total, 210 post-smolts of Atlantic salmon of strain Stofnfiskur Iceland (SF Optimal), with an average weight of 110.9 g, were used in the challenge, which took place approximately 1 yr after hatching. The fish were unvaccinated and pre-screened (at 5 and 15 g of size) and tested negative for SAV, infectious salmon anaemia virus (ISAV), infectious pancreatic necrosis virus (IPNV), piscine myocarditis virus (PMCV), piscine orthoreovirus (PRV) and salmon gill poxvirus (SGPV).

Parent fish were pre-screened for the equivalent, apart from SGPV.

2.3. SAV3 inoculum

The SAV3 originated from pooled heart and head kidney samples of Atlantic salmon from the Hordaland region of Norway (Taksdal et al. 2015). Propagation of SAV3 was performed using the CHSE-214 cell line (ATCC® CRL-1681™), which was derived from a Chinook salmon *Oncorhynchus tshawytscha* embryo. These cells were grown on L-15 (Leibowitz) medium (Lonza), supplemented with 10% fetal bovine serum (FBS) and gentamicin at 20°C. Serial 10 fold dilutions of the SAV3 stock (passage number 4) were inoculated onto 24 h old CHSE-24 monolayers in 96 well plates, allowing quantification. The viral endpoint titre, measured as 50% tissue culture infective dose ($TCID_{50}$), was determined to be 10^6 $TCID_{50}$ ml^{-1} as described by Reed & Muench (1938).

2.4. Experimental tanks

Three identical 500 l seawater tanks were used in the challenge trial, designated T_L, T_H and T_C for low viral dose, high viral dose and negative control tank, respectively. The seawater originated from 105 m depth from Byfjorden and had been filtered through 20 µm drum filters and treated with UV light (135 W m^{-2}). The water flow in all tanks was the same throughout the experiment, with an average flow rate of 950 l h^{-1} $tank^{-1}$ and was set according to the biomass, dissolved oxygen level and tank water temperature in order to meet an optimal oxygen level for the fish. Water was monitored daily for temperature, salinity and dissolved oxygen levels throughout the challenge. During the challenge period in T_L, T_H and T_C, respectively, the following variations were seen; dissolved oxygen ranged between 79–97, 80–97 and 79–86%, tank water temperatures between 11.7–12.3, 11.7–12.3 and 11.5–12.4°C, and salinity levels between 34.1–34.5, 34.1–34.5 and 34.2–34.5‰.

All tanks had a daily photoperiod of 12 h light:12 h dark, provided by an automatic artificial lighting system. During the 12 h of light, an automatic feeder dispenser fed the fish with 3 mm Nutra Olympic pellets (Skretting). The amount of food given in T_C was between 80–150 g; in T_L, between 56–140 g; and in T_H, between 56–140 g; amounts were adjusted marginally as the fish were growing, dying or being sampled.

Clinical signs in the fish, as well as mortality, were monitored daily in all 3 tanks. Dead experimental fish were removed daily and did not undergo any further analysis.

2.5. Challenge

A total of 45 fish (shedder fish) were immersed into a bath with the anaesthetic Finquel® vet. 1000 mg g^{-1} (100 mg l^{-1}). Once immobilized, each shedder fish was administered with 0.2 ml inoculum by intraperitoneal (i.p.) injection of either a low SAV3 dose of 2×10^2 $TCID_{50}$ $fish^{-1}$, a high SAV3 dose of 2×10^4 $TCID_{50}$ $fish^{-1}$ or virus-free Leibovitz-15 (L-15) cell culture medium containing 2% FBS (mock inoculum). All shedder fish were marked by adipose fin clipping, which allowed us to distinguish them from cohabitant fish. Each group of 15 shedder fish was then transferred into the respective 500 l seawater tank (T_L, T_H and T_C) containing 55 cohabitant fish which had been transferred into the tanks 2 d earlier (–2 d post-challenge, DPC). The shedder fish remained in the tanks throughout the entire challenge period. The challenge was performed and approved in accordance with the Norwegian Animal Research Authority (NARA).

2.6. Sampling

Sampling of tank water and cohabitant fish was performed at 16 different time points: 0, 7, 12, 16, 19, 20, 21, 22, 23, 24, 25, 28, 29, 30, 33 and 37 DPC. Water sampling was carried out by using sterilized 1000 ml Borosilicate 3.3 glass bottles (VWR®) that were submerged by hand to approximately 15 cm below the water surface with the mouth of the bottle turned towards the water current. Five 1 l water samples were taken from each of the 3 tanks at each sampling time point. T_C was sampled first, followed by T_L and lastly T_H.

Three fish were randomly collected from each of T_L and T_H, and one fish was collected from T_C at each sampling point. Sampled fish were euthanized through an immersion bath with an overdose of Finquel® vet. 1000 mg g^{-1} (150 mg l^{-1}). Gross pathology was evaluated, and tissue samples were collected from heart (including the valves and bulbus arteriosus) and mid-kidney for RT-qPCR (stored in RNA*later*™ Soln. [Thermo Fisher Scientific Baltics], at room temperature) and heart and pancreas (pyloric caeca with attached pancreatic tissue) for histopathology (stored in 10% neutral buffered formalin, at room temperature).

2.7. Histopathology

Histopathological examination was performed with a light microscope on pancreas and heart tissue samples from cohabitant fish, identified as SAV3-positive by RT-qPCR, in order to confirm SAV infection and PD. The tissue samples were fixed in 10% neutral buffered formalin and processed according to standard procedures at the Norwegian Veterinary Institute in Oslo (NVI, Oslo).

2.8. Concentration of SAV3 from tank water samples

Concentration of SAV3 from 1 l water samples from each of the 3 water tanks was performed according to 5 CMs, previously developed by Weli et al. (2021). Briefly, the methods involved filtration of 1 l tank water through either an electropositive or an electronegative membrane filter, followed by elution of the adsorbed material from the filter with one of 4 different buffer solutions (buffer 1–4). An overview of the 5 filter/buffer combinations is given in Table 1. Filters were then inserted into a 47 mm in-line filter holder (Merck Millipore) fitted to a Masterflex® portable environmental sampler pump (Cole-Parmer Instrument Company). Following filtration of the water sample, the filter was rinsed using a buffer solution.

For the methods using buffer 1, the intact filter was rinsed in a 50 mm Petri dish with 2.4 ml buffer 1 and subsequently shaken on an orbital shaker at 600 rpm for 30 min. For the 3 other buffer solutions, the filter was cut into <1 cm^2 fragments and transferred into a 50 ml CELLSTAR® tube (Greiner Bio-One) containing 4.0 ml buffer 2, 3 or 4 and subsequently vortexed 3 times for 1 min with 5 min rests. The eluate (i.e. tank water concentrate) was distributed as 1 ml into 1.5 ml Eppendorf® SafeSeal tubes (Sarstedt AG & Co.) and stored at −80°C until RNA extraction.

Tank water from T_C was filtered first, followed by T_L and lastly T_H. Hoses and filter heads were disinfected between sampling of each tank by pumping (flow rate 0.2 l min^{-1}) 1 l of 10% chlorine for 10 min, followed by neutralisation with 1 l of 10% Alfa Aesar sodium thiosulfate pentahydrate, 99+% (Thermo-Fisher Scientific) for 10 min and finally by rinsing with 1 l distilled water for 10 min.

2.9. RNA extraction of tank water concentrates

One ml NucliSENS® easyMAG® Lysis Buffer (bioMérieux) was added to 1 ml tank water concentrate followed by RNA extraction, using the easyMAG® robot (bioMérieux) and the standard lysis protocol (generic 2.0.1.), which was performed with 50 µl magnetic silica beads, according to the NucliSENS easyMAG user manual (bioMérieux 2009). RNA was eluted in 50 µl buffer and stored at −80°C until use in RT-qPCR.

2.10. RNA extraction from organ tissue

Tissue samples (i.e. heart and mid-kidney) were stored in RNA*later*™ Soln. (Thermo Fisher Scientific Baltics) at −80°C prior to RNA extraction, which was performed by adding approximately 20 mg tissue with 180 µl ATL Lysis Buffer (Qiagen®) and 20 µl Proteinase K and incubation overnight at 56°C. Extraction was performed with QIAcube® (Qiagen®) with the reagents from the DNeasy Blood & Tissue Kit (Qiagen®), giving an RNA elution volume of 200 µl. Isolated RNA was stored at −80°C until use in RT-qPCR.

Table 1. Overview of the 5 different concentration methods ($CM_{A–E}$) used to concentrate salmonid alphavirus subtype 3 (SAV3) from tank water during the cohabitant challenge trial with post-smolt Atlantic salmon. The methods are presented as 5 different combinations of membrane filters and buffer solutions used for SAV3 adsorption and elution, respectively

Concentration method	Filter	Buffer no.	Buffer solution
CM_A	Electronegative[a]	1	Lysis buffer[c]
CM_B	Electropositive[b]	1	Lysis buffer[c]
CM_C	Electronegative[a]	2	1 mM NaOH[d] (pH 9.5)
CM_D	Electronegative[a]	3	L-15 medium[e] (pH 9.0) + 2% FBS
CM_E	Electropositive[b]	4	L-15 medium[e] (pH 7.3–7.75) + 2% FBS

[a]Electronegative MF-Millipore™ 0.45 µm MCE membrane (Merck Millipore); [b]Electropositive Zeta Plus™ 1 MDS membrane (3M Purification); [c]NucliSENS® easyMAG® Lysis Buffer (bioMérieux); [d]1 mM NaOH (Sigma-Aldrich); [e]L-15 (Leibovitz) Medium (Lonza)

2.11. RT-qPCR

The SAV3 strain was detected using the Q_nsP1 assay (Hodneland & Endresen 2006). This broad-spectrum assay detects all known SAV subtypes using primers and probe with final concentrations of 500 and 300 nM, respectively and amplifies a conserved region in the 5' end of the Q_nsP1-gene, giving amplicons of 107 bp (Table 2).

Extracted RNA was automatically pipetted by Eppendorf epMotion® 5075 (Eppendorf) in duplicates, analyzed by RT-qPCR on an AriaMx machine (Agilent Technologies) and evaluated with the Agilent AriaMx Real-Time PCR software (version 1.7). Each plate included a negative control sample and an inter plate calibrator of pure SAV3 RNA, which were both run in duplicates.

The cut-off quantification cycle (Cq) value was set to 40; samples with values below this Cq in duplicates were considered positive. Samples with only one positive parallel were rerun and considered positive only with positive duplicates. The template volume was 2.0 µl RNA in a total reaction volume of 20 µl, and the RT-qPCR kit used was TaqMan® Fast Virus 1-Step Master Mix (Applied Biosystems®). The thermal programme comprised reverse transcription for 5 min at 50°C and enzyme activation for 2 min at 95°C, followed by 45 cycles of 15 s at 94°C and 40 s at 60°C. GraphPad Prism 4.03 (GraphPad Software) was used to plot the data.

2.12. Check of RNA purity

All tank water concentrates and tissue samples were evaluated for inhibitors that could impact the RT-qPCR and target quantification. RNA was, therefore, analyzed undiluted (1:1) and diluted (1:4) in duplicates, by RT-qPCR. Potential inhibition was detected when the Cq difference between the 1:1 and 1:4 samples was found to be less than 2 Cq. For these samples, the 1:4 dilution was used to estimate virus quantities.

2.13. Quantification of SAV3

A sample of purified SAV3 RNA (inter plate calibrator) was quantified by RT-ddPCR. The RT-ddPCR analysis was performed as described by Weli et al. (2021), by using the One-step RT-ddPCR Advanced Kit for Probe (Bio-Rad Laboratories), and the primers and probe used for the RT-qPCR assay, with the final concentrations of 900 and 250 nM, respectively.

A 2-fold serial dilution (1:1 to $1:2^{12}$) was run by RT-qPCR as a standard curve. The amplification efficiency (E = 94%), correlation coefficient (r^2 = 0.995) and slope of the linear regression line were all evaluated in accordance with Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Taylor et al. 2010).

Quantification of SAV3 particles was performed based on the following formula: $N_1 = N_2 \times (1 + E)^{(Cq_1 - Cq_2)}$ (Christensen et al. 2017), where N_1 and N_2 represent the SAV3 copy number in the unknown sample and the calibrator, respectively and Cq_1 and Cq_2 represent the SAV3 detection in Cq values in the unknown sample and the calibrator, respectively. In order to estimate the number of viral particles in 1 l of tank water concentrated with the first CM (CM_A), copy numbers were multiplied with $25 \times 2.4 \times 1/R$ where R (recovery) is approximately 25%, as calculated according to Weli et al. (2021). The data were $\log_{10}$ transformed and plotted in GraphPad Prism 4.03 (GraphPad Software).

2.14. Limit of detection and limit of quantification of SAV3

The limit of quantification (LOQ) and limit of detection (LOD) values for the RT-qPCR were calculated using the following formulas: $LOQ = 10\sigma/S$ and $LOQ = 3.3\sigma/S$, where σ is the standard deviation of y-intercepts from 2 SAV3 standard curves and S is the slope of the curve, according to the ICH Q2 (R1) guidelines (EMEA 1995). LOD and LOQ for SAV3 in 1 l of tank water, using the CM_A method, was estimated as described in Section 2.13.

Table 2. The Q_nsP1 assay was used for the detection of salmonid alphavirus subtype 3 (SAV3) in tissue samples and tank water concentrates, from the cohabitant challenge trial with post-smolt Atlantic salmon

Oligonucleotide	Sequence	Position	Amplicon length (bp)	Reference strain	Reference
Q_nsP1 F primer	5'-CCG GCC CTG AAC CAG TT-3'	17–33	107	AY604235	Hodneland & Endresen (2006)
R primer	5'-GTA GCC AAG TGG GAG AAA GCT-3'	54–69			
Probe	FAM-5'-CTG GCC ACC ACT TCG A-3'-MGB	103–123			

2.15. Statistical comparisons of Cq values

In order to determine whether there was a statistically significant difference in performance between the 5 CMs, pairwise comparisons of the CMs were performed in one analysis (Model 1), and between the 2 membrane filters and the 4 buffer solutions with another (Model 2). Post hoc Tukey's HSD tests were used to achieve this goal. The performance was measured as Cq values from the RT-qPCR. Cq values were log transformed prior to the regressions to approximate normality. Cq values above 40 (i.e. no detection of SAV3 RNA) may constitute a problem for the assumption of normality, and were therefore removed. The virus was only detected in 2 observations in T_L using CM_E and was therefore clearly not suited to extract SAV3 RNA in this case, hence it was removed from the data set. Non-linear temporal autocorrelation was handled by modelling sampling time points (i.e. DPC) as a third-degree polynomial, since common approaches like a first-order autoregression (AR1) and autoregressive moving-average (ARMA) are not warranted for sampling designs with uneven sampling intervals. The inclusion of DPC will handle the development of the disease over time, as well as the removal of fish for tissue analysis. A forward model selection procedure was used to evaluate the inclusion of explanatory variables, using Akaike's information criterion (AIC) as an optimization criterion, and interactions were evaluated after the fixed effects were included. The best and the next best models were compared using an ANOVA table function in R. Explanatory variables, tried in the model selection for Model 1, were DPC (as a third-degree polynomial), viral dose and CM. Explanatory variables tried in model selection for Model 2 were DPC (as a third-degree polynomial), viral dose, filter and buffer. The model was validated using a cross validation procedure: the data set was randomly split into training data (80 % of the data set) and validation data (20 %). The model was refitted on the training data and used to predict the Cq values in the validation data set; predicted and observed validation data was stored. This procedure was repeated 10 000 times. Predictive R^2 values were calculated as the squared Pearson correlation coefficient between observed and predicted Cq values, and were used as a measure of how well the model is able to predict observations removed from the data set, in order to evaluate model overfitting in the linear models.

Associations between log-transformed Cq values in tank water concentrates and tissue (heart or midkidney) in both viral dose tanks (T_L and T_H) were estimated using a mixed effect linear model approach (Model 3) and backwards model selection, using AIC as an optimization criterion. The best and the second best models were compared using an ANOVA table function in R. Temporal autocorrelation and repeated measures (3 fish analyzed from each tank at each sampling time point) were handled by including DPC as a random intercept term, and viral dose tanks (T_L and T_H) as a fixed effect term. As a single water sample was analyzed by each CM at each sampling time point, sampling repeatability was deducted from the variability in the statistical model. This was done by simulating 1000 data sets from the model (using the 'simulate()-' function in R), and investigating the distribution of the simulated samplings.

All analyses were performed using the R statistical software (version 3.6.2), and multiple comparisons (Tukey's HSD test) based on the linear model were fitted using the 'multcomp' package (Hothorn et al. 2008). Mixed effect models were fitted using the 'lme4' package (Bates et al. 2015).

3. RESULTS

3.1. Clinical signs

Clinical signs associated with PD included an overall reduced appetite (i.e. failure to thrive), lethargy and abnormal swimming behavior and findings of faecal casts and mucus in the tank water. These signs were observed for the first time at 12 DPC in the high viral dose tank (T_H) and at 16 DPC in T_L (Fig. 1).

3.2. Mortality

One dead cohabitant fish was observed in T_L, at 20 DPC, whereas no cohabitant fish died in T_H until the last day of the challenge (1 fish died at 37 DPC; Fig. 1). In T_C, no fish died during the challenge period.

3.3. Gross pathology

Characteristic gross pathological changes associated with PD in cohabitant fish, including yellow mucoid gut content, empty intestines, ascites and petechial haemorrhages in the fat surrounding the pyloric caeca, were observed for the first time at 16 DPC in T_H and at 20 DPC in T_L, and thereafter

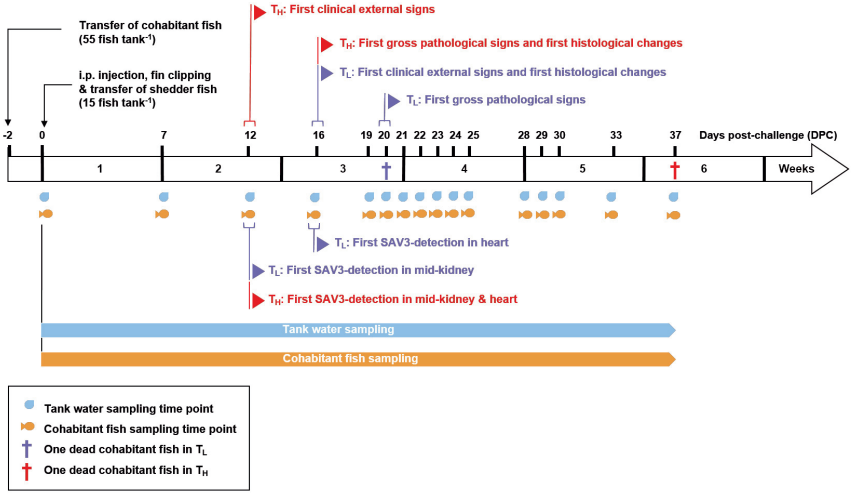

Fig. 1. Timeline for the salmonid alphavirus subtype 3 (SAV3) cohabitant challenge trial with post-smolt Atlantic salmon showing the 16 sampling time points, given as days post-challenge, for tank water and cohabitant fish. At Day 0, shedder fish were administered with a 0.2 ml SAV3 inoculum by intraperitoneal (i.p.) injection and subsequently fin clipped prior to their distribution between the 3 different tanks: the low viral dose tank (T_L: 2×10^2 50 % tissue culture infective dose [$TCID_{50}$] $fish^{-1}$), the high viral dose tank (T_H: 2×10^4 $TCID_{50}$ $fish^{-1}$) and the negative control tank (T_C: virus-free L-15 [Leibovitz] medium)

observed at every sampling time point in almost all sampled fish in both T_L and T_H. Additionally, cohabitant fish collected from T_L had a more severe gross pathology associated with PD in the beginning of the challenge period compared to fish collected from T_H. For comparison, cohabitant fish from T_C were also evaluated and had normal autopsy findings throughout the challenge.

3.4. Histopathology

Tissue samples from the cohabitant fish collected from T_L and T_H started presenting typical histopathological signs associated with SAV infection at 16 DPC, including necrosis and severe loss of exocrine pancreatic tissue and focal myocardial degeneration, evaluated by haematoxylin and eosin (H&E) staining (except for the cardiac tissue sections from 3 cohabitants collected from T_L at 16 DPC). Different levels of histopathological changes typical of SAV infection were found at all later sampling time points. For comparison, cohabitant fish collected from T_C were evaluated at all sampling time points and presented intact and normal pancreatic and cardiac muscle tissues. The pancreatic tissue sections from the cohabitant fish sampled from T_L and T_H between 16, 29 and at 37 DPC (one cohabitant fish from T_L) were positive by immunohistochemistry (IHC) staining for SAV, indicating acute SAV infection on these days.

3.5. Detection of SAV3 in tissue samples

A total of 94 tissue samples (2 were lost) from cohabitant fish from each of T_L and T_H, and 32 from T_C were analyzed by RT-qPCR. A majority were found to contain inhibition. Inter-individual variability was observed in the concentration of SAV3 RNA copy numbers detected in the tissue samples (mid-kidney and heart), in both T_L and T_H. SAV3 was first detected in T_H at 12 DPC for both mid-kidney (Cq 27.9) and heart (Cq 27.0) (Fig. 2B), and in T_L at 16 DPC for both mid-kidney (Cq 19.0) and heart (Cq 21.1) (Fig. 2A). Peak levels of SAV3 detection in the mid-kidney were seen at 19 DPC in T_L (Cq 14.7) and at 16 DPC in T_H (Cq 19.9) (Fig. 2A,B). Peak levels of SAV3 detection in the heart were seen at 23 DPC in T_L (Cq 15.0) and at 21 DPC in T_H (Cq 14.9) (Fig. 2A,B). On the last sampling time point (37 DPC), the highest SAV3 concentration was detected in the heart in T_L (Cq 17.7), followed by the mid-kidney in T_L (Cq 23.0), the heart in T_H (Cq 23.1) and the mid-kidney in T_H (Cq 28.3). SAV3 was detected in all tissue samples from the onset until (and including) the last day of challenge (37 DPC) in both T_L and T_H.

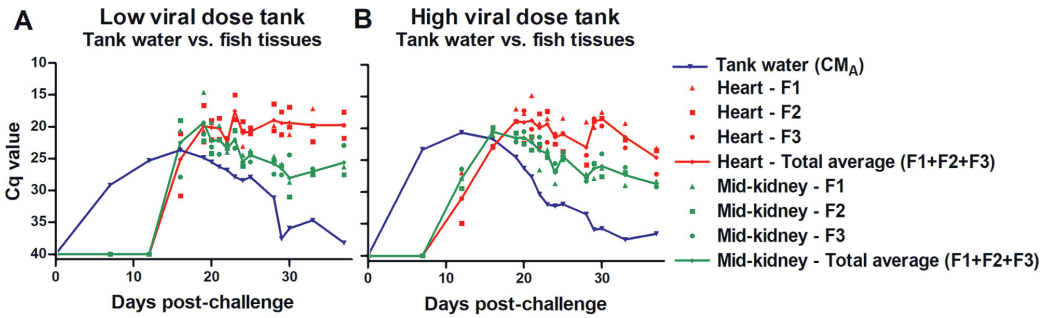

Fig. 2. Comparison between detection of salmonid alphavirus subtype 3 (SAV3) in 1 l tank water concentrated with method A (CM_A) and in tissue samples (mid-kidney and heart) from cohabitant fish sampled during the SAV3 cohabitant challenge. The samples were analysed by RT-qPCR. The SAV3 concentration is presented as the average quantification cycle (Cq) values per days post-challenge in the (A) low viral dose tank (T_L) and (B) high viral dose tank (T_H). Tank water samples were collected simultaneously with the collection of the 3 cohabitant fish $tank^{-1}$ (F1: fish 1; F2: fish 2; F3: fish 3) at 16 sampling time points, from both T_L and T_H

Tissue samples collected from T_C were found to be negative by RT-qPCR.

3.6. Detection of SAV3 in tank water samples

Eighty 1 l water samples were collected from each of the 3 tanks (T_L, T_H and T_C), resulting in a total of 240 samples that were analysed by RT-qPCR, and the majority of these samples were found to contain inhibitors. The first detection of SAV3 was made on the second sampling time point (7 DPC) in both T_L and T_H, with all CMs except CM_E.

3.7. Comparison of the 5 CMs ($CM_{A–E}$)

Compared with the other 4 CMs, Fig. 3 revealed a general tendency for CM_A, the combination of electronegative filter with buffer 1, to be best in recovering SAV3 from 7 DPC until the end of challenge. This tendency can be observed in both T_L and T_H, but with a stronger tendency in the latter (Fig. 3A,B). Additionally, the period of virus detection was narrower and had a higher detection peak in the T_H compared to T_L (Fig. 3C,D). When using CM_A, the first SAV3 detection in tank water was made at 7 DPC in both T_L and T_H; the peak of viral shedding occurred earlier in T_H (12 DPC), with 3.42×10^6 SAV3 RNA copies l^{-1} (6.53 $\log_{10}$ copies l^{-1}), than in T_L (16 DPC), with 4.7×10^5 SAV3 RNA copies l^{-1} (5.67 $\log_{10}$ copies l^{-1}) (Fig. 3C,D). Water samples collected from T_C were negative, except for one sample collected at 19 DPC (Cq 34.6), when using CM_D. The simulated samplings from the statistical model showed that the Cq values varied between 19.7 and 45.0 for CM_A in both tanks over the entire course of the challenge. More than 99% of these simulated samplings returned Cq values below 40, indicating that we would have detected SAV in more than 99% of the samples at any time point after 7 DPC.

The best fitted model for CM (Model 1) included a CM, DPC as a third-degree polynomial (allowing non-linear time trends in the development of the infection during the challenge period) and viral dose (ANOVA model comparison: $p < 0.001$). The model also included interactions between CM and viral dose, and between viral dose and DPC, allowing a slower development of the infection and a lower peak in T_L. The model became quite complex, and overfitting was a serious concern. The predicted R^2 from cross validation (0.78) was close to the multiple R^2 (0.84), proving that the model can predict observations that were excluded from the training data with good precision. Pairwise comparisons revealed that CM_A returned significantly lower Cq values than all the other CMs when SAV3 was detected in T_H (Table 3; CM_A: p-values between 0.004 and <0.001), but CM_A was not different from CM_B in T_L ($p = 0.988$).

The best fitted model for filter and buffer (Model 2) included filter, buffer, DPC (as a third-degree polynomial) and viral dose, in addition to interactions between viral dose and DPC, and between viral dose and filter (ANOVA model comparison: $p < 0.001$). The predicted R^2 (0.78) was close to the multiple R^2 (0.84), proving that the model can predict observations that were excluded from the training data with

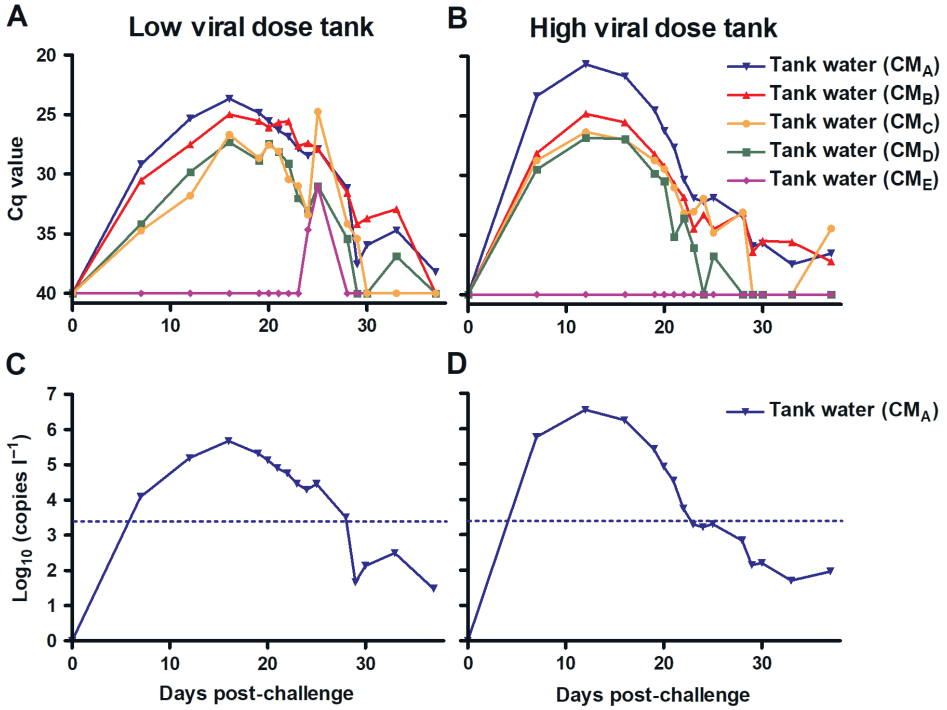

Fig. 3. Detection of salmonid alphavirus subtype 3 (SAV3) from tank water sampled at 16 time points, given as days post-challenge (DPC) throughout the SAV3 cohabitant challenge trial with post-smolt Atlantic salmon. The samples were analyzed by RT-qPCR. Five methods ($CM_{A–E}$) were used to concentrate SAV3 from 1 l of tank water in the low viral dose tank (T_L) and high viral dose tank (T_H). Both (A,B) quantification cycle (Cq) values per DPC and (C,D) SAV3 copies l^{-1} of tank water DPC^{-1} are presented. Dotted horizontal line: limit of quantification when using CM_A

good precision. Pairwise comparisons revealed that buffer 1 performed significantly better than buffers 2 and 3 ($p < 0.001$ for both comparisons), and the electronegative filter performed significantly better than electropositive for T_H ($p = 0.002$), but not for T_L ($p = 0.91$) (Table 3).

3.8. LOD and LOQ of SAV3 in 1 l of tank water

The LOD and LOQ when using CMA were 902 and 2736 SAV3 RNA copies l^{-1}, respectively.

3.9. Detection of SAV3 in tank water concentrates vs. tissue samples

Associations between virus recovery in tissue samples and tank water concentrates were explored using a linear mixed effect model (Model 3). The model was fitted using a backwards model selection (AIC). The full model (before model selection) explained the tank water concentrates' Cq values for SAV3 detection as the effect of DPC (as a random intercept term), viral dose (as a factor variable), tissue samples' Cq values for SAV3 detection and the interaction between the Cq values for tissue samples and dose. Any removal of explanatory terms gave a poorer fit, and the full model was therefore kept (confirmed by the ANOVA model comparison, $p = 0.02$). The model output is presented in Table 4. A significant positive association was established between the Cq values for mid-kidney samples and tank water concentrates ($p = 0.0084$) in both T_L and T_H. The effect of viral dose and the interaction were significant ($p < 0.038$ and $p = 0.024$, respectively). No significant effect was found with a similar approach for heart tissue samples ($p = 0.26$).

Table 3. Post hoc Tukey HSD tests, based on the linear models (Models 1 and 2), exploring how the quantification cycle (Cq) values for the detection of salmonid alphavirus subtype 3 (SAV3) could be explained by the concentration method (CM), the 2 membrane filters (electropositive or electronegative) and the 3 buffer solutions (buffers 1, 2 and 3) used for the concentration of SAV3 from 1 l tank water. The columns indicate the null hypotheses, the mean differences and the p-values. Note that there was an interaction between CM and viral dose in both models. Tukey comparisons (contrasts) in these cases are shown for detected SAV3

Tukey null hypotheses	Mean difference of log(Cq)	p
CM in high viral dose tank (model 1)[a]		
$CM_B - CM_A = 0$	0.073	0.004
$CM_D - CM_A = 0$	0.16	<0.001
$CM_C - CM_A = 0$	0.092	<0.001
$CM_D - CM_B = 0$	0.088	0.003
$CM_C - CM_B = 0$	0.019	0.820
$CM_C - CM_D = 0$	–0.069	0.040
CM in low viral dose tank (model 1)[a]		
$CM_B - CM_A = 0$	–0.0069	0.988
$CM_D - CM_A = 0$	0.098	<0.001
$CM_C - CM_A = 0$	0.087	0.001
$CM_D - CM_B = 0$	0.10	<0.001
$CM_C - CM_B = 0$	0.094	<0.001
$CM_C - CM_D = 0$	–0.011	0.968
Filter and buffer (model 2)[a]		
Electropositive – Electronegative, high viral dose	–0.064	0.002
Electropositive – Electronegative, low viral dose	0.0023	<0.909
Buffer 1 – Buffer 3 = 0	–0.13	<0.001
Buffer 2 – Buffer 3 = 0	–0.036	0.101
Buffer 2 – Buffer 1 = 0	0.089	<0.001

[a]See Table 1

4. DISCUSSION

In the present study, we present a successful CM for detection of SAV3 in seawater. We have previously demonstrated the potential of this method for recovery of SAV3 spiked into natural seawater (Weli et al. 2021). However, in order to optimise the method for the field, 5 CMs ($CM_{A–E}$), based on 2 different membrane filters and 4 different buffer solutions, were evaluated for their ability to recover SAV3 from tank water collected during a cohabitant challenge trial, when post-smolt Atlantic salmon had developed clinical infection with SAV.

The method combining the electronegative membrane filter and NucliSENS® easyMAG® Lysis Buffer (CM_A) had the highest SAV3 recovery and the most consistent SAV3 detection in tank water samples from T_H throughout the challenge, but virus recovery using CM_A or CM_B was indistinguishable in samples from T_L. This buffer solution and the electronegative membrane filter performed significantly better compared to the other buffer solutions and the electropositive filter used herein, although the filters were indistinguishable in samples from T_L. This conclusion is also consistent with the above-mentioned *in vitro* study, in which we concluded that the same combination of the electronegative filter and Nucli SENS® easyMAG® Lysis Buffer provided the best SAV3 recovery from seawater (Weli et al. 2021). The method combining the electropositive filter and NucliSENS® easyMAG® Lysis Buffer (CM_B) also resulted in higher SAV3 recovery compared to the other 3 CMs (CMc, CM_D and CM_E), thus strengthening the importance of the NucliSENS® easyMAG® Lysis Buffer, as stated by our previous *in vitro* study (Weli et al. 2021).

Horizontal transmission of SAV via water has been confirmed by other experimental challenge studies, in which SAV was detected directly from seawater using filtration (Andersen et al. 2010, Jarungsriapisit et al. 2016a,b, 2020). In these studies, an electropositive 1 MDS filter was used for filtration of 1 l seawater, followed by rinsing of the filter using lysis buffer (Andersen et al. 2010, Jarungsriapisit et al. 2016b) or L-15 supplemented with 10% FBS (Jarungsriapisit et al. 2016a, 2020). In other words, none of these studies used the filter/buffer combination (CM_A) that proved most successful in the present study. However, in this context it is important to highlight that the subsequent processing of the filtrate (i.e. RNA extraction) and the PCR assay used can also impact on the method's success in detecting SAV3 from seawater.

Although the cohabitation challenge model, as used in this study, does not represent the natural route of infection (since the initiation of infection was done via i.p. injection of shedder fish), it does simulate spread of the virus and a natural exposure route to the cohabitant fish.

McLoughlin & Graham (2007) suggested that the incubation period for SAV may be 7–10 d at seawater temperatures of 12–15°C. In the present study, the tank water temperature was approximately 12°C, and SAV3 was first detected in the tank water at 7 DPC, compared to in cohabitant fish tissue at 16 DPC in T_L and at 12 DPC in T_H. However, the early detections of SAV3 in the tank water may have been

Table 4. The most parsimonious model explaining the association between quantification cycle (Cq) values for detection of salmonid alphavirus subtype 3 (SAV3) in the mid-kidney samples and in the tank water concentrates. Only fixed effects from the model are shown. Note that Cq values are log transformed in the response; '×' means interaction. Baseline for the model output is high dose. This means that the intercept and log(mid-kidney × Cq-values) from the table are parameters in a linear function between log(tank water Cq values) and log(mid-kidney Cq values) in samples from the high viral dose tank. In this case the model takes the form '$a + bx$', where 'a' is the intercept estimate and 'b' (the slope) is the log(mid-kidney Cq values) estimate. The estimate for low viral dose is presented as the deviation from the intercept for high viral dose, and the 'log(mid-kidney Cq values) × low viral dose' estimate is presented as the deviation from the slope for high viral dose. The 'a' in the linear function for low viral dose becomes the intercept + low viral dose estimates, and 'b' is the log(mid-kidney Cq values) + log(mid-kidney Cq values) × low viral dose estimates

Variable	Estimate	SE	p
Intercept	2.7	0.27	< 0.001
log(mid-kidney Cq values)	0.22	0.081	< 0.008
Low viral dose	−0.51	0.24	< 0.038
log(mid-kidney Cq values) × low viral dose	−0.17	0.076	0.024

virus excreted from the shedder fish that remained in the tanks throughout the challenge.

In contrast to the *in vitro* study (Weli et al. 2021), the present study involved filtration of tank water that contained a high amount of organic matter (e.g. faecal casts, mucus and pellets). Furthermore, the tank water samples were collected approximately 15 cm below the water surface of the tanks, and a previous study (Stene et al. 2016) has identified accumulated levels of SAV in surface lipids due to fat droplets leaking from dead and SAV-infected fish. Hence, the detected SAV3 from the tank water in the present study could have been influenced by the inevitable inclusion of the fat droplets and/or organic matter (e.g. faecal casts and mucus) that have been found positive for SAV3 RNA, as shown by previous research (Graham et al. 2011).

In the present study, SAV3-infection in cohabitant fish was confirmed by gross pathology, histopathology and clinical signs associated with PD (Nelson et al. 1995, McLoughlin et al. 1996, 2002, Taksdal et al. 2015, Jansen et al. 2017), as well as by RT-qPCR analysis.

Heart and mid-kidney, which are the recommended organs for SAV detection according to the World Organisation for Animal Health (OIE) and are used in the Norwegian surveillance program (Lovdata 2017), were sampled from cohabitant fish in the present study. A significant positive correlation was found throughout the challenge between the levels of SAV3 in the tank water samples and in the mid-kidney samples collected from the cohabitant fish in both T_L and T_H. This observation supports that the cohabitant fish provided a significant contribution to virus levels in the tank water throughout the challenge. No correlation was found between virus concentrations in the tank water samples and the heart samples, either in T_L or in T_H. However, the virus concentrations were generally found to be higher in the heart samples compared to the mid-kidney.

Tank water samples were collected consistently from all 3 tanks before the collection of the cohabitant fish, ensuring that the fish would not be exposed to stress that might cause them to increase their shedding rate prior to the water sampling. The water flow rate in all 3 tanks was constantly high (950 l h^{-1}), ensuring not only optimal oxygen levels for the fish, but also providing self-cleaning of the tanks throughout the challenge. Hence, it is highly suspected that the one water control which was found to be SAV3-positive when using CM_D was due to cross-contamination during sampling or sample processing.

Monitoring for the presence of SAV3 in the tank water sampled during the challenge period was done by RT-qPCR, which enables high performance in detection of waterborne RNA viruses at low concentrations (Girones et al. 2010, Rački et al. 2014a,b). RT-qPCR has been reported to be more sensitive to inhibitors than qPCR (Girones et al. 2010), which may cause lower quantification precision (i.e. larger coefficients of variation) (Rački et al. 2014a). Since the present study involved sampling of natural seawater, in which both salts and other RT-qPCR inhibitors are expected to have an influence on RNA-virus quantification, a check of inhibition was made for all tank water samples, and inhibition was indeed present in a majority of the samples (hence the 1:4 dilution was considered for these). Therefore there is an advantage of using RT-ddPCR on these samples, as this method is less sensitive to any effect of inhibitors (Rački et al. 2014a,b). The method is more expensive compared to RT-qPCR, but the samples could then be run on undiluted RNA only.

The SAV3 concentrations (based on Cq values) in tissue samples were higher overall compared to in-tank water samples. This is an expected finding because the fish (in contrast to the water) serve as a replication site for the virus. However, in this context, it is important to highlight that the detection method

used for tissue is different than the method used for water, and therefore these methods are not comparable in terms of differences in the amount of detected virus.

In laboratory as well as in field studies, the sensitivity of the detection methods should be evaluated according to whether the method is able to detect the virus when there are SAV3-infected fish in the population. In order to compare the sensitivity of the water sampling method to a surveillance method based on sampling of fish under field conditions, the comparison will be between the LOD for the water sampling method on the one side, and the probability of sampling an infected fish on the other. For both methods, the sensitivity will obviously depend on the prevalence of SAV3-infected fish in the population. However, a water sample serves as a representation of the environment of a large number of fish, and if the water sampling method can detect the virus at a low prevalence of SAV in the fish population, it may increase the probability for early detection of SAV in a fish farm.

Based on these findings, the combination of an electronegative charged filter (MF-Millipore™ 0.45 µm MCE membrane; Merck Millipore) and NucliSENS® easyMAG® Lysis Buffer (bioMérieux) is considered to have the best potential in serving as a more cost-efficient, straightforward, rapid, reliable, reproducible and animal-welfare friendly method for concentration and detection of SAV3 and potentially other SAV subtypes from seawater. This new method will be tested for surveillance of farmed salmonid populations as a part of a biosecurity plan for SAV under natural field conditions. The method might allow warning and earlier implementation of disease control measures on farms neighbouring farms with identified SAV, which would be of significance in Atlantic salmon health management.

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Paper III

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Title: Early detection of salmonid alphavirus in seawater from marine farm sites of Atlantic salmon (*Salmo salar*)

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Running page head: Surveillance of salmonid alphavirus in seawater.

ABSTRACT

The traditional strategy for national surveillance of salmonid alphavirus (SAV) infection in Norwegian fish farms relies on a costly, time-consuming, and resource-demanding approach based on the monthly sampling of fish from all marine farms with salmonids. In order to develop an alternative surveillance method, a water filtration method was tested in parallel with the ongoing surveillance program in seven Norwegian marine farm sites of Atlantic salmon (*Salmo salar* L.) with no current suspicion of SAV infection. During the period from May 2019 to January 2020, seawater samples were collected from the top layer water inside all net-pens at seven sites, and concentrated for SAV by filtration through an MF-Millipore™ electronegative membrane filter, followed by rinsing with NucliSENS® Lysis Buffer, before RNA extraction and analysis by RT-qPCR. SAV was detected from seawater at an earlier stage compared to traditional sampling methods, in all sites where the fish tested positive for SAV. A significant negative temporal relationship was observed at all sites between the SAV concentration found in seawater samples and the number of days until SAV was detected in the fish. This means that, the fewer SAV particles in the seawater, the more days it took until SAV was detected in fish samples. Based on this, sampling of seawater every month for the surveillance of SAV has a great potential as an alternative method for early detection of SAV in Atlantic salmon farms.

Keywords: Surveillance, water filtration, SAV detection, SAV concentration, salmonid alphavirus, pancreas disease, RT-qPCR, field study.

1. INTRODUCTION

Water-transmitted viral pathogens are significant threats to aquaculture, challenging fish welfare and the economy of this industry. They have been difficult to control due to, e.g. an increased susceptibility among hosts and limited understanding of the transmission dynamics (Kibenge 2016). One of the most serious pathogens in sea-farmed salmonids, with regards to the fish welfare and economic impact, is salmonid alphavirus (SAV) (Jansen et al. 2015, Sommerset et al. 2020). SAV is a small (~ 12 kb genome), enveloped, positive-sense single-stranded RNA virus (+ssRNA), and it is the aetiological agent of pancreas disease (PD) (Weston et al. 2002, Hodneland & Endresen 2006).

The first report on PD was made in farmed Atlantic salmon (*Salmo salar*, L.) in Scotland in 1976 (Munro et al. 1984). In Norway, it was first described in 1989 (Poppe et al. 1989) and became a notifiable disease (list 3) in 2007. A total of six subtypes of SAV (SAV1-SAV6) are known (Fringuelli et al. 2008). Two of these are known to Norwegian aquaculture, i.e. SAV2 and SAV3, forming two separate PD endemic zones with marine SAV2 in the Northwest- and Mid-Norwegian coast, and SAV3 along the southwestern coast (Hjortaas et al. 2016, Sommerset et al. 2020).

In 2017, the national surveillance program for PD was intensified, introducing a PD zone for SAV2 and SAV3 (i.e. Western- and Mid-Norway), and two surveillance zones north and south/south-east of the PD zone (Lovdata 2017). This program requires monthly sampling of fish from all SAV-negative marine operative farm sites with Atlantic salmon, rainbow trout (*Oncorhynchus mykiss*, Walbaum) and char (*Salvelinus alpinus*), in order to reduce the consequences of the disease within the defined PD-zones, as well as to prevent further spread of SAV. The strategy for surveillance of SAV infection in fish farms relies on a time-consuming and resource-demanding approach, involving monthly sampling of ~ 20 fish from each of these sites and analyzing heart tissue from each fish by reverse-transcription quantitative real-time

PCR (RT-qPCR). Histopathological investigations should then follow to confirm the diagnosis of PD. This screening aims at representing the SAV status for the whole farm site population. However, when SAV is recently introduced into a population, the chances of sampling infected fish are small, given that there are probably few infected individuals.

Transmission of SAV occurs horizontally, through the water (McLoughlin et al. 1996, Graham et al. 2007, 2012, Kristoffersen et al. 2009, Aldrin et al. 2010, 2015, Xu et al. 2012). We, therefore, suggest that SAV shed from the fish into their aquatic environment, could be detected in seawater sampled from the net-pens, and that seawater samples might be more representative of the infection status for the farm site population. Moreover, monitoring of waterborne pathogens through filtration of water is an animal-friendly method, making the sacrifice of fish redundant, and with a potential for earlier implementation of disease control measures (Strand et al. 2014, 2019, Rusch et al. 2018, 2020).

Experimental studies have successfully used filtration as a way of detecting SAV in seawater (Andersen et al. 2010, Jarungsriapisit et al. 2016a,b, 2020). Recently, we carried out an *in vitro* study, involving filtration of artificial and natural seawater that was spiked with SAV3 (Weli et al. 2021), and next a SAV3 cohabitant challenge trial with Atlantic salmon post-smolts (Bernhardt et al. 2021). In both these studies, the best method for concentration and detection of SAV was filtration through an electronegative membrane filter with subsequent rinsing of the filter with a lysis buffer.

In the present study, the aim was to test this method in the field to monitor the presence of SAV in Norwegian marine farm sites of Atlantic salmon, which had no detection of SAV in fish prior to the trial. Water monitoring was performed in parallel with the mandatory surveillance program.

2. MATERIALS AND METHODS

2.1. Pilot study design

In August 2018, a pilot study was conducted to evaluate the applicability of the filtration method to detect SAV in seawater. Samples were collected from a farm site that was found positive for SAV approximately three months earlier under the surveillance program. This site had five floating open marine net-pens with Atlantic salmon, which had been sea transferred in August and September 2017, in the Hordaland county (Note that Hordaland and Sogn and Fjordane counties were merged into the new Vestland county, on January 1st, 2020) in Western Norway (inside the SAV3 endemic zone).

In this study, seawater sampling was carried out from one net-pen (Ø: 50 m) enclosing a number of ~ 180 000 Atlantic salmon with an average weight of ~ 2.5 kg. One litre (1 L) seawater samples were collected in duplicate from three sampling depths (0.15 m, 5 m, and 10 m) and at three sampling points (A, B, and C; Fig. 1). A rubber dinghy was inserted inside the net-pen to collect water from sampling points A and B, which were located on diametrically opposite sides inside the net-pen, ~ 8 m from the inner edge, while sampling from point C was done from a service boat, ~ 8 m from the outer edge of the net-pen. The major ocean current direction was from sampling point A to B to C.

Top layer samples were obtained by immersing the open 1 L sterilized plastic bottle (VWR®, Norway) by hand down to a depth of 0.15 m, allowing the water to fill the plastic bottle. For the other two sampling depths (i.e. 5 m and 10 m), a Ruttner 2 L Standard Water Sampler (Hydro-Bios, Germany) was used. The filtration of the seawater samples took place on-site. Seawater temperature (˚C) was measured at the three sampling depths on the day of sampling by using an automated monitoring system at the site.

2.2. Large-scale field study design

In May 2019, a large-scale field study was initiated involving monthly seawater sampling over nine months, ending in January 2020. Seven sites (S_A-S_G) of Atlantic salmon inside the SAV3 endemic zone on the Western Norwegian coastline, in the counties of Vestland (formerly Hordaland) and Rogaland (N-S: 55 km; E-W: 42 km) (Fig. 2), were selected, based on their recorded positive PD history in earlier fish generations during the years 2013-2018 (records from https://www.barentswatch.no/fiskehelse). Fish in five of these sites (i.e. S_A, S_D, S_E, S_F and S_G) had been transferred to the sea in autumn 2018, while the fish in the remaining two sites (i.e. S_B and S_C) were sea transferred in spring 2019. S_B and S_C, and S_D and S_E are neighbouring sites with a sea way distance (i.e. the shortest way between the sites in water, around island, peninsulas or other hindrances in the water) of ~ 3 km and ~ 2.7 km apart, respectively. S_F and S_G are situated with a sea way distance of ~ 10.7 km. S_B and S_C, S_D and S_E and S_F and S_G were sampled on the same day at every occasion.

There was no recorded presence of SAV in fish from any of these sites by the start of this study. Fish health inspectors from a single fish health service performed the fish sampling from all sites (S_A-S_G) every month, accordingly with the national surveillance program for SAV. In connection with this, one 1 L seawater sample was collected from every net-pen at each of the seven sites (i.e. 6, 5, 4, 6, 10, 6 and 7 net-pens initially at S_A, S_B, S_C, S_D, S_E, S_F and S_G, respectively); typically once a month and occasionally twice (i.e. at the beginning and the end of the month).

2.2.1. Seawater sampling

The sampling strategy for the large-scale field study was based on the results from the pilot study and was similar at all seven sites. One litre of seawater was collected inside each net-pen at a site, using a 1 L sterilized plastic bottle (VWR®, Norway), which was vertically

positioned to a bottle holder, attached to a 65-120 cm telescopic swing sampler (Bürkle GmbH, Germany). The bottle was swung horizontally while submerged approximately 0.15 m below the water surface, close to the net-pen's inner side. On occasion, a 1 L seawater sample was collected from each site from 0.15 m below the water surface, at a randomly decided sampling point located ~ 200 – 300 m away from the site (September-October 2019).

Following sampling, all bottles from one site were wrapped with bubble wrap insulation and stored surrounded by an abundance of cooling elements in a 45 L Proxon cooler box (Pinnacle, Tokyo Plast International Ltd., India). The samples were sent to the laboratory of the Norwegian Veterinary Institute (NVI, Oslo, Norway) by express delivery (within 24 h), were measured for their temperature at arrival and thereafter stored at 4 °C prior to filtration, which was started immediately.

For each sampled site, a registration form was filled in by the fish health inspector and added to each shipment, including information about any abnormal observations (e.g. unstable weather conditions, algal bloom, increase in fish mortality, behavioural changes such as abnormal swimming patterns and reduced appetite, and handling procedures carried out, like transfer of the fish populations between net-pens on the location due to, e.g. treatment). The seawater temperature (°C) for each site was measured at 3 m depth on the same week as the seawater sampling took place (data retrieved from http://www.barentswatch.no/fiskehelse/).

2.3. Concentration of seawater samples

The concentration of 1 L seawater samples was performed as previously described (Weli et al. 2021, Bernhardt et al. 2021). Filtration was performed by using a 47 mm electronegative charged membrane filter (MF-Millipore™ 0.45 µm MCE membrane, Merck Millipore, USA) into a 47 mm in-line filter holder (Merck Millipore, USA). The 1 L seawater samples were filtered using a peristaltic pump (V6-3L Peristaltic Pump, Schenchen, China) at a flow rate of

200 mL min^{-1}. After filtration, the filter was placed upside down onto a 50 mm petri dish containing 2.4 mL NucliSens® Extraction Buffer 1 (easyMAG®, bioMérieux, Marcy l'Etoile, France) for the pilot study, and NucliSENS® Lysis Buffer for the large-scale field study. The Petri dish was shaken on an orbital shaker (600 rpm) for 30 min. The seawater concentrate was aliquoted (1 mL), stored at -80 °C until RNA extraction and analyzed by RT-qPCR.

A process control with a known quantity of *Mengovirus* strain MC_0 (type strain: ATCC VR-1957) was added into each 1 L seawater sample from the large-scale field study before filtration.

2.4. Extraction of RNA

The RNA extraction was done by using the easyMAG® robot (bioMérieux, Marcy l'Etoile, France) and the standard lysis protocol (generic 2.0.1.) with 50 µL magnetic silica beads, according to the NucliSENS easyMAG user manual (bioMérieux S.A. 2009). A mixture of 1 mL seawater concentrate and 1 mL NucliSENS® Lysis Buffer was extracted to a volume of 40 µL buffer. In the pilot study, the total volume of concentrate was extracted (2 mL). The RNA was stored at -80 °C until analysis by RT-qPCR.

2.5. RT-qPCR

Detection of SAV RNA in both the pilot and large-scale field study, was made by RT-qPCR, as previously described (Weli et al. 2021). A broad-spectrum Q_nsP1 assay that targets the nsP1-gene in SAV, giving amplicons of 107 base pairs (bp) (Hodneland & Endresen 2006), was used. Each primer had a final concentration of 500 nM (R-primer: 5'-GTA GCC AAG TGG GAG AAA GCT-3' and F-primer: 5'-CCG GCC CTG AAC CAG TT-3') and a final concentration of 300 nM of the probe (FAM-5'-CTGGCCACCACTTCGA-3'-MGB).

Detection of the *Mengovirus* was performed using the final concentration of 500 nM of each primer and a final concentration of 500 nM of the probe (ISO 2017).

The reverse transcription quantitative real-time PCR (RT-qPCR) kit used was TaqMan® Fast Virus 1-Step Master Mix (Applied Biosystems, Foster City, CA, USA), with a total reaction volume of 20 µL and a template volume of 2 µL. The cycle conditions of the RT-qPCR comprised reverse transcription for 5 min at 50 °C, denaturation for 2 min at 95 °C, followed by 40 cycles of 15 s at 94 °C and 40 s at 60 °C.

For each plate, a negative control sample, an inter-plate calibrator (IPC) of stock SAV RNA and *Mengovirus* RNA, corresponding to the amount spiked in the seawater samples, were included. Samples from the field study were run in triplicates, while samples from the pilot study were run in duplicates, by RT-qPCR. In cases of some parallels giving a negative result, samples were rerun in triplicates before considered as positive.

2.6. Quantification of SAV copies

Quantification of SAV was done by using the following formula: $N_1 = N_2 \cdot (1+E)^{(Cq_2 - Cq_1)}$ (Christensen et al. 2017), where N_1 and N_2 are the SAV copy numbers in the sample and the IPC, respectively, E the amplification efficiency, and Cq_1 and Cq_2 are the Cq values for the sample and the IPC, respectively. The IPC was quantified using RT-ddPCR, performed as previously described (Weli et al. 2021).

A SAV standard curve was prepared with a series of two-fold dilutions run by RT-qPCR, which gave an E of 0.94, a correlation coefficient (r^2) of 0.995, and a slope (s) of -3.475, which fulfil the requirements of the MIQE guidelines (Taylor et al. 2010).

The number of viral particles in 1 L of seawater was estimated by multiplying the copy number in 2 µL RNA with $20 \cdot 2.4 \cdot \frac{1}{R}$, where R is the recovery approximated to be 0.25 (25 %),

as calculated by Weli et al. (2021). The SAV data was $\log_{10}$-transformed before plotted using Excel (Microsoft Office Professional Plus 2016, USA).

Mengovirus was not quantified, but run as an internal process control to monitor the methodology's performance. The samples were analyzed undiluted (1:1) and diluted (1:4) to check for the presence of inhibitors (e.g. salt and organic matter) that could influence the RT-qPCR and target quantification. Undiluted samples were considered as being inhibited if a Cq difference of less than 2 cycles was found between the 1:1 and 1:4 dilutions. For these samples, the 1:4 dilution was used to estimate the virus quantities.

2.7. Fish analysis

Farmed Atlantic salmon investigated for SAV were collected monthly in connection with the national surveillance program for SAV/PD (i.e. $\geq$ 20 fish/site) in all seven sites. Samples of the spongious tissue from the cardiac ventricle were stored in RNAlater™, processed and analyzed for SAV by RT-qPCR at external laboratories. Positive results were reported to the Food Safety Authorities and published on Barentswatch (https://www.barentswatch.no/fiskehelse).

2.8. Sequencing of SAV in seawater samples

A selection of the SAV-positive seawater concentrates was applied for Sanger sequencing of the SAV E2 gene (which allows separation between all known SAV subtypes), on an Applied Biosystems® 3500xl Genetic Analyzer (Foster City, CA) with BigDye Terminator v3.1 Cycle Sequencing Kit, according to standard protocols at NVI (Oslo, Norway). Primers identical to those previously used in the PCR amplification step were used for the sequencing (F-primer: 5'-GCCACCACCTGTCCGATCTG-3'; R-primer: 5'-ACCAAGGTTCCGTGTAGTTAGC-3'), giving amplicons of 488 bp (Hjortaas et al. 2013).

2.9. Statistical analysis

An investigation was carried out to examine whether there was a temporal relationship between the number of SAV copies detected in the seawater, and the number of days until SAV was detected in heart tissue from fish.

Days between detections are bound by the generations at the sites that were monitored. Therefore, if SAV is not detected at a site during a generation, this may be because the fish population has not been infected, or because it was slaughtered before the viral shedding gave detectable amounts of viral RNA; the time between SAV-detections is unknown in the latter case. This so-called "right-censored" data is common in data used for survival analyses. Here, we employed a survival regression under the assumption that the errors were following the Weibull distribution. In addition, the number of copies recovered using RT-qPCR has a limit of detection (LOD). If we do not detect viral RNA in the sample, this may be either because the virus was not present, or present in concentrations below LOD. In this case, the data was "left-censored", which can be an issue with a large amount of non-detects. To overcome this issue, we imputed data below LOD as random draws from a lognormal distribution, where the mean and standard deviation was derived from a censored data using the Kaplan-Meier method (Canales et al. 2018, Lee 2020). The number of copies was ln transformed to approximate normality.

An eventual relationship between days and copy numbers L^{-1} may differ between the sites, due to differing oceanographic properties. This was tested by including the site as a factor variable in the model selection. Samples were collected from all net-pens at each site and considered as pseudo-replicates. Therefore, we included only the mean SAV copy number L^{-1} from each site sampled at the same time in the analyses. Model selection was performed using the Akaike's information criterion (AIC), and competing models were tested using the analysis

of variance tables (Anova function in R). Level of significance (α) for all analyses was set to 0.05.

All statistical analyses were performed using the R statistical software (version 3.6.2) (R Core Team 2019). The survival regression was performed using the survival package (Therneau & Grambsch 2000, Therneau 2015). The model was plotted using the ggplot2 package in R (Wickham 2016).

3. RESULTS

3.1. Pilot study

A total of eighteen seawater samples collected in the pilot study, were all found to contain SAV, with 75 % showing an inhibitory effect on the RT-qPCR. The parallel SAV measurements at three water depths and sampling points differed but showed the same trend in sampling points A and B (Fig. 3).

Inside the net-pen (i.e. sampling points A and B), the SAV concentrations were descending with the water depths. Overall, the highest SAV concentrations were detected in the seawater samples collected inside the net-pen at 0.15 m below the water surface, with 5.91 $\log_{10}$ (copy number L^{-1}) and 5.97 $\log_{10}$ (copy number L^{-1}) from sampling points A and B, respectively. The SAV concentrations at sampling points A and B at 0.15 m depth were approximately at the same level.

Outside the net-pen (i.e. sampling point C), the SAV-detection revealed the opposite trend to sampling points A and B, with the highest SAV concentration at 10 m (5.08 $\log_{10}$ [copy number L^{-1}]).

From the sampling depths 0.15 m, 5 m and 10 m, the seawater temperatures were 17.3 °C, 17.2 °C, and 16.9 °C, respectively.

3.2. Field study

A total of 286 seawater samples were collected from all net-pens at seven different Norwegian marine farm sites of Atlantic salmon (S_A-S_G) during May 2019 – January 2020 (Table 1). Additionally, seven seawater samples were collected at a distance of ~ 200-300 m from each site (September-October 2019). The sample temperature was measured to be between 0.3-18.8 °C (7.0 ± 3.4 °C), upon arrival at NVI (Oslo, Norway). Forty-two samples (15 %) were SAV-positive, of which 25 of these (60 %) showed an inhibitory effect. Thirteen of the SAV-positive samples (31 %) were above LOQ, while the remaining 29 (69 %) were below LOQ and of these 25 were below LOD. The results for each site are shown in Table 1.

At S_A, SAV was first detected in the seawater in June 2019 from one of the six net-pens, i.e. four months before the first SAV detection was made in heart tissue from fish (Table 1). The following month no detection of SAV was made in the seawater samples collected from any of the net-pens, however, the presence of the virus was again revealed from August till December 2019. A monthly increase of the SAV concentration in the seawater collected at the site between August and November was followed by a decrease in all four net-pens in December. In January 2020, no SAV detection was made in any of the seawater samples collected from the site. The highest SAV concentration (5.34 $\log_{10}$ [copy number L^{-1}]) was found in the seawater sample collected from one of the four net-pens in November. PD was first confirmed by analysis of fish samples collected at S_A in October 2019. The initial number of net-pens at S_A was six. Of these, the fish populations in two net-pens were slaughtered in August and in January, respectively before water sampling, leaving only two net-pens for collection of seawater samples the last month of the field study.

Low concentrations of SAV were only found in seawater from two out of five net-pens at S_B in late August (i.e. second sampling that month), and in October from one out of the four net-pens at S_C. SAV was never detected in fish from S_B during the study period, or any time

before slaughter. At S_C, SAV was detected in fish in June 2020, i.e. after the study period had ended.

Seawater collected at S_D revealed the presence of SAV only once during the study period, which was in one out of five net-pens in October. At S_E, SAV was detected in the seawater in one out of ten net-pens as early as in June (with a comparable SAV concentration to what was found at S_D in October), and then every second month with an increasing virus concentration. Detection of the highest SAV concentration in seawater collected from S_E was made in October. SAV was never detected in fish samples at S_D during the study period, or any time after until fallowing, while at S_E, PD was confirmed in February 2020.

At S_F, SAV was detected in the seawater for the first time in July from one out of six net-pens, next in all five net-pens in November, and then in one out of two and in two out of two in December and January, respectively. The highest concentration of SAV was found in seawater collected in November and SAV was found in fish samples in January. At S_G, SAV was detected in the seawater samples for the first time in August in one out of the seven net-pens, and next in January in two out of the five remaining net-pens, which were found positive with the highest SAV concentrations at this site during the study period. SAV was never detected in fish at S_G during the study period, or any time after until fallowing.

One 1 L seawater sample was collected ~ 200 – 300 m from each site in September (i.e. S_A, S_B, S_F and S_G) and in October (i.e. S_C, S_D and S_E), and only one of these (S_A) revealed presence of SAV by RT-qPCR. At S_A, S_D, S_F and S_G, the number of net-pens decreased due to slaughtering of fish populations, leaving fewer net-pens for sampling for the study period.

The seawater temperatures measured at 3 m depth on the same weeks as the sampling, varied between 8.1-17.2 °C (11.6 ± 2.9 °C) at S_A, between 8.0-17.7 °C (13.0 ± 3.4 °C) at S_B, between 8.0-16.7 °C (11.5 ± 2.9 °C) at S_C, between 11.9-18.4 °C (14.7 ± 2.9 °C) at S_D, between

11.6-18.2 °C (14.4 ± 2.9 °C) at S_E, between 6.5-19.0 °C (12.7 ± 4.1 °C) at S_F and between 6.7-18.0 °C (12.8 ± 3.8 °C) at S_G.

3.4. Sequencing of SAV from the seawater samples

Three seawater samples with the highest SAV concentrations from different sites (i.e. S_A, S_F, and S_G), were selected for sequencing of the E2 gene. The sequence from S_A (Cq < 30) represented SAV3, while seawater samples from the other two sites (i.e. S_F: Cq > 30 and S_G: Cq > 35), did not give any useful sequences.

3.5. Statistical analysis

During the large-scale field study, SAV was detected in both the 1 L seawater- and fish samples at four out of the seven sites (i.e. S_A, S_C, S_E, and S_F), and SAV was detected earlier in the seawater than in fish at all sites. A significant negative relationship could be observed between the SAV copy number and number of days between SAV-detection in seawater and in fish samples at the same site (Fig. 4 and Table 2). This means, with a higher SAV copy number detected in the seawater samples, the closer in time was SAV detection in fish.

As we can see from Fig. 4, SAV copies were quantifiable only in S_A, and much of the trend in the model was therefore driven by the data from S_A. A new reduced model was therefore fitted on data only from S_A and used in a sensitivity analysis. The reduced model was qualitatively identical to the full model including all sites (data not shown).

4. DISCUSSION

This study demonstrated that SAV can be detected earlier in seawater samples than in fish samples collected monthly from different Norwegian marine farm sites of Atlantic salmon. We have shown that the filtration method has a great potential as an alternative method for

surveillance of SAV in fish farms. The result is consistent with our previous *in vitro* and challenge trial studies (Weli et al. 2021, Bernhardt et al. 2021).

First, a pilot study was carried out in a net-pen holding a fish population with a recent PD-diagnosis, with the aim to find out at which water depth and location the highest concentrations of SAV in seawater could be found, as a way of optimizing for the following large-scale field study. Seawater samples collected from the top layer (0.15 m) and greater depths (5 m and 10 m) did not show large differences in SAV concentrations. However, sampling from the top layer also constituted by far the most practical method to be applied under field conditions and gave support to the choice of the sampling procedure for the large-scale field study.

In the main study, we compared surveillance of SAV in marine farm sites of Atlantic salmon, by use of sampling of seawater versus screening of fish. Results showed that detection of SAV was made earlier in seawater than in fish samples in all sites where the fish tested positive for SAV (i.e. S_A, S_C, S_E, and S_F). Thus, water sampling from a fish population's aquatic environment seemed to give a more representative image of the SAV status and an earlier detection of the virus, than a sampling of relatively few fish individuals.

A significant increase in SAV copies was found in the seawater samples, the closer in time SAV-detection was made in fish (Fig. 4). However, the results showed that most of the SAV-positive seawater samples were below LOD, which could indicate that the filtration method or the screening design need some further adjustments, such as filtering more samples each month, larger volumes (e.g. 5 L) or implementing more frequent collection of samples from all net-pens. On the other hand, the fact that so many of the seawater samples were SAV-positive by RT-qPCR, but below LOD, indicates that the LOD, which was estimated in a challenge trial with tank water, is too high for the seawater samples in the present study. Namely, the actual recovery of the virus will vary from sample to sample. We may have had a

recovery for our samples that was higher than the 25 % as calculated according to the *in vitro* study (Weli et al. 2021), meaning our LOD is a "conservative" estimate, which does not overestimate the sensitivity of the method. LOD and LOQ were not calculated for the field water samples due to practical reasons.

In the present study, SAV detection in the seawater was done at sites with varying temperatures, but the highest SAV concentrations were found in the colder months (Table 1). This observation is in contrast to the monthly incidence rate for PD in salmonids in Norway, which showed the highest incidence during the summer and autumn (Sommerset et al. 2020). In fact, previous observations suggest that temperature at the time of infection may be connected to the pathogenesis of PD; when temperatures are rising, more acute and shorter-lived outbreaks occur, while when temperatures are declining, there are more chronic courses of the outbreaks (McLoughlin & Graham 2007). Hence, in the present study, the higher SAV concentrations found in the colder months could be due to more shedders, or coincidences such as the time of withdrawal, sampling location, etc. but primarily due to the fact that the virus particles are more stable the colder the water (Graham et al. 2007, Jarungsriapisit et al. 2020).

Consistent seawater sampling was ensured for the large-scale study by a team of fish health inspectors, with each inspector assigned their own specific task. Before the start of the field study, they had all received on-site instructions and educational material, including a manual, instructional video recording and equipment, ensuring consistency in the way the seawater samples were collected and stored. The sampling of fish was done in order to comply with the mandatory surveillance program, was carried out according to the regulations, and as such, not under the control of the present study. Hence, there is no information about which net-pen the sampled fish were withdrawn from, in connection with the monthly sampling.

Natural seawater knowingly contains both salts and other RT-qPCR inhibitors which might influence the virus quantification. In this study, most of the SAV-positive seawater

samples showed an inhibitory effect, which was solved by using the 1:4 dilution for quantification of SAV in these samples. Diluting the RNA is not desirable as detection sensitivity and the precision of quantification are reduced. This problem could have been avoided by using reverse-transcription droplet digital PCR (RT-ddPCR), which has a higher tolerance to inhibitors (Rački et al. 2014a,b). However, RT-ddPCR is a more expensive method, and due to a large number of seawater samples in the present study, RT-qPCR was used for detecting SAV. The molecular method for detection of SAV is based on the selected segment of the virus's genetic material, meaning SAV detected in the seawater does not necessarily have to be viable. Nevertheless, this study aimed to provide information about the mere presence of the virus in seawater from the aquatic environment of the farmed fish.

Seawater samples were transported by express delivery to the laboratory of NVI (Oslo, Norway) for filtration and analysis. However, a more convenient and cheaper use of the method, especially if increasing the water volume, can be achieved by processing the sample in the field and subsequently shipping the concentrate stored at -20°C (preferably -80°C). Alternatively, the filter could be stored in some form for transport medium (e.g. lysis buffer). Overall, there is a need for optimization of the method and screening design during more studies, including more extended study periods, under different environmental conditions and testing sampling procedures, in order to establish water filtration as an alternative method for surveillance of SAV at fish farms.

Based on these findings, it was shown that the filtration method has great potential to be implemented for surveillance of farmed salmonid populations for early detection of SAV infection. It seems to be a straightforward, cost-efficient, time-saving, resource-saving, and an animal welfare-friendly approach for detecting this virus at fish farms, compared to traditional methods. If seawater samples are identified as SAV-positive in connection with surveillance, then infection in the fish population will still have to be confirmed by subsequent sampling and

testing of fish. Nevertheless, this approach for surveillance will reduce to a great extent the overall sacrifice of fish, and also the costs connected to it. Earlier detection can also initiate subsequent implementation of disease control measures on neighbouring farms, allowing improved biosecurity measures and more effective marine-farmed Atlantic salmon health management. The water analysis may also have the potential for surveillance of other pathogens in farmed fish populations.

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Table 1. Overview of the SAV detection analysed by reverse transcription quantitative real-time PCR (RT-qPCR) in 1 L seawater samples and in fish collected on the same day during May 2019 – January 2020, in connection with the national surveillance program for *salmonid alphavirus* (SAV)/pancreas disease (PD). Samples were collected from all the net-pens at seven different sites (S_A-S_G) of Atlantic salmon (*Salmo salar* L.) that were sea transferred either in autumn 2018 (A-2018) or in spring 2019 (S-2019). SAV-positive samples are marked in green. For seawater, the number of SAV-positive samples out of the total samples is given and the quantifiable SAV concentrations (given as $\log_{10}$[copy number L^{-1}]), are shown. For fish, SAV-positive and SAV-negative heart samples, are shown as "+" and "-", respectively (notified to the Food Safety Authorities, records from https://www.barentswatch.no/fiskehelse/). The last column shows the official SAV / PD status for each site from the start of the field study until the fallowing.

Site	Sea transfer	Type of sample	May 2019	June 2019	July 2019	Aug 2019	Sept 2019	Oct 2019	Nov 2019	Dec 2019	Jan 2020	Last month of fish harvest	SAV / PD status
S_A	A-2018	Seawater	0/6	1/6	0/6	2/4	3/4	3/4 [3.83-4.87]	4/4 [4.92-5.34]	4/4 [4.31-4.56]	0/2	Jan 2020	Oct 2019 (PD-diagnosis)
		Fish	-	-	-	-	-	+	+	+	nd		
S_B	S-2019	Seawater	0/5	0/5	0/5	0/5 | 2/5	0/5	0/5	0/5	0/5	0/5	July 2020	None
		Fish	-	-	-	-	-	-	-	-	-		
S_C	S-2019	Seawater	*nd*	0/4	0/4	0/4	*nd*/4 ¤	1/4	0/4	0/4	0/4	June 2020	June 2020 (SAV in fish)
		Fish	*nd*	-	-	-	-	-	-	-	-		
S_D	A-2018	Seawater	*nd*	0/6	0/6	0/6	0/5	1/5	*nd*	*nd*	*nd*	Dec 2019	None
		Fish	*nd*	-	-	-	-	-	*nd*	*nd*	*nd*		
S_E	A-2018	Seawater	*nd*	1/9*	0/10	2/10	0/10	5/10	*nd*	*nd*	*nd*	March 2020	Feb 2020 (PD-diagnosis)
		Fish	*nd*	-	-	-	-	-	*nd*	*nd*	*nd*		
S_F	A-2018	Seawater	*nd*	0/6	1/6 | 0/6	0/6	*nd*/6 ¤	0/6	5/5 [3.52-3.82]	1/2	2/2	Jan 2020	Jan 2020 (SAV in fish)
		Fish	*nd*	-	-	-	-	-	-	-	+		
S_G	A-2018	Seawater	0/7	0/7	0/7	1/7	*nd*/7 ¤	0/7 | 0/6	0/5	*nd*/5 ¤	2/5	Feb 2020	None
		Fish	-	-	-	-	-	-	-	-	-		

Note: * 9 of 10 seawater samples were analyzed by RT-qPCR; *nd* no data; ¤ no seawater sampling was performed at the time point given, from any of the net-pens at the site (due to either oblivion, i.e. S_C, or late sampling on the previous month, i.e. S_F and S_G).

Table 2. The model selection revealed that the number of days between *salmonid alphavirus* (SAV) detection in seawater and fish, was an effect of the farm site and the copy number detected in the 1 L seawater samples. Therefore, the final model included days between detections as a response variable, the copy number per L in the seawater samples, and the site as predictors. The estimates for Site A (S_A) is the intercept, while the other sites are presented as differences from the intercept (contrasts).

	Estimate	Std. Error	Significance (p < 0.05)
Intercept - Site A (S_A)	4.70	0.28	p < 0.001
Site B (S_B)	16.8	7780	p = 1
Site C (S_C)	1.55	0.37	p < 0.001
Site D (S_D)	15.7	11500	p = 1
Site E (S_E)	1.22	0.41	p = 0.003
Site F (S_F)	0.78	0.37	p = 0.034
Site G (S_G)	15.8	9140	p = 1
Number of copies (log)	-0.26	0.058	p < 0.001
Log(scale)	-0.33	0.16	p = 0.039

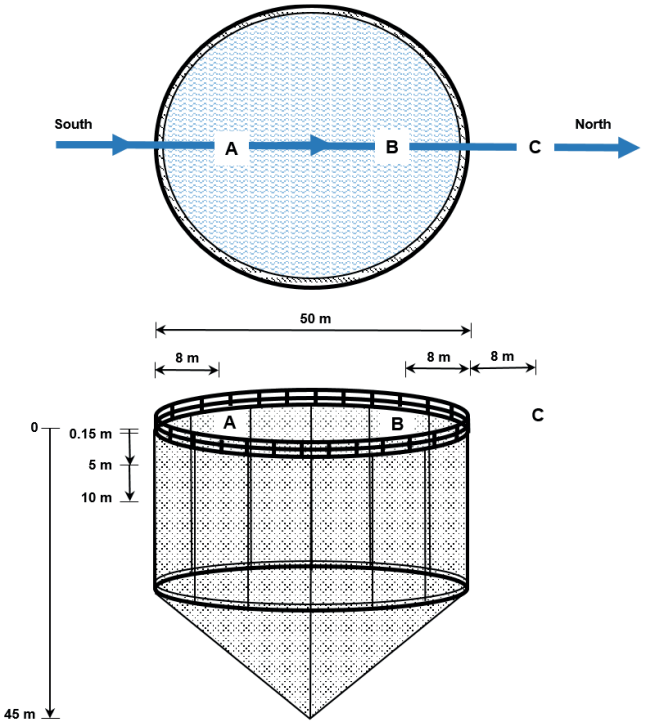

Fig. 1. Schematic illustration of the sampling design for the pilot study of detection of *salmonid alphavirus* (SAV) in seawater samples. Duplicate 1 L seawater samples were collected from three different depths (0.15 m, 5 m, and 10 m) at three different sampling points (A, B, and C) in relation to one net-pen. The major ocean current direction was from sampling point A to C and is represented by the blue horizontal arrow on the top image. Note: The measurements are not to scale.

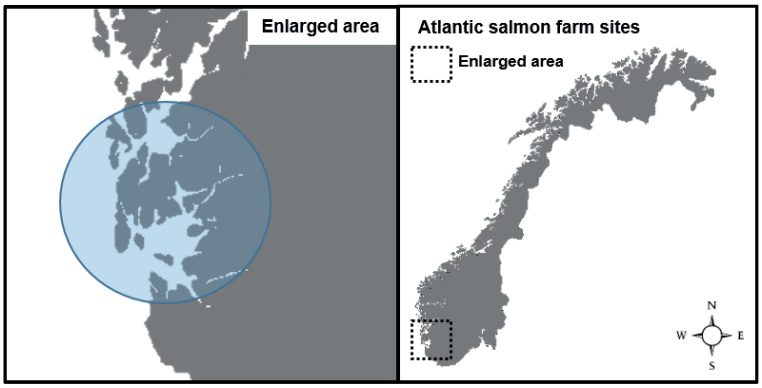

Fig. 2. The area holding the seven Norwegian marine farm sites (S_A-S_G) of Atlantic salmon (*Salmo salar* L.) which were sampled for seawater during May 2019 – January 2020. (Edit of the original map, retrieved from https://www.vemaps.com).

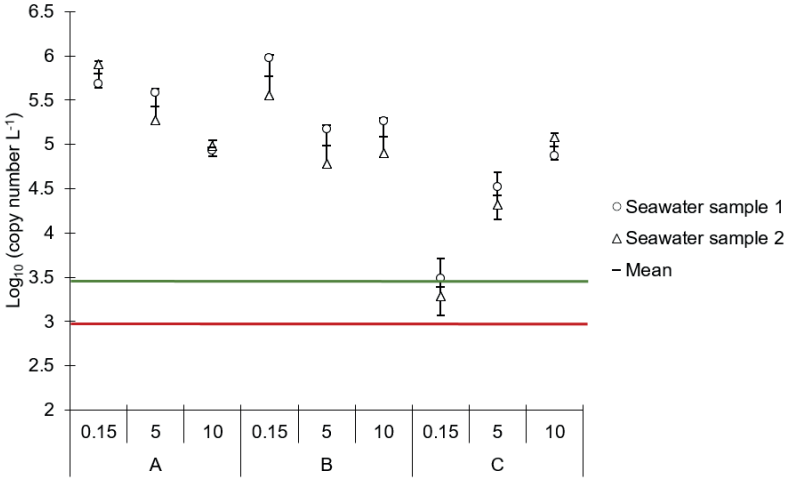

Fig. 3. Log-transformed *salmonid alphavirus* (SAV) copy numbers L^{-1} in seawater from a net-pen holding pancreas disease diagnosed fish (pilot study), analyzed by reverse transcription quantitative real-time PCR (RT-qPCR). Nine seawater samples were collected in duplicates (2 x 1 L) at three different sampling depths (0.15 m, 5 m, and 10 m) and from three different sampling points, A and B located on diametrically opposite sides inside the net-pen, ~ 8 m from the inner edge, and C located outside the net-pen, ~ 8 m from the outer edge. The SAV concentrations of the two seawater replicates, i.e. seawater sample 1 ("○") and seawater sample 2 ("△") are shown (log_{10} [copy number L^{-1}]), together with the mean of both ("-") and the standard deviation of the mean represented by the error bars. The limit of detection (LOD: 2.94 log_{10} [copy number L^{-1}]) and the limit of quantification (LOQ: 3.44 log_{10} [copy number L^{-1}]) for the RT-qPCR, as calculated by Bernhardt et al. (2021), when using the filtration method and considering the 25 % recovery, are represented by the solid red and green horizontal lines, respectively.

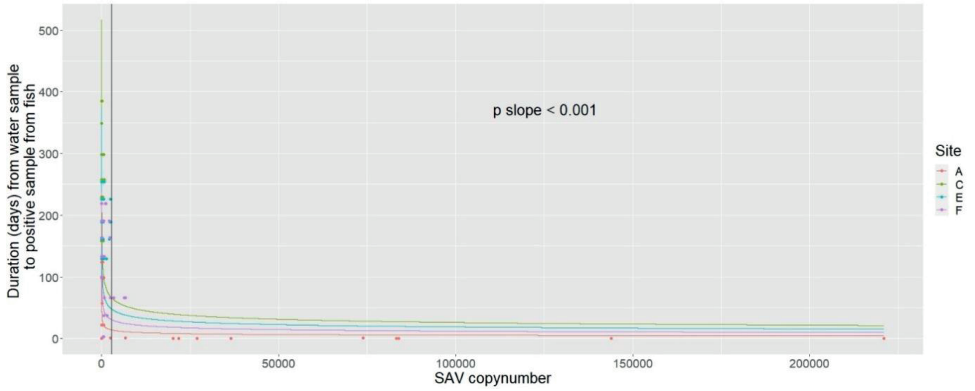

Fig. 4. Relationship between the number of *salmonid alphavirus* (SAV) copies (predictor) in 1 L seawater samples and the number of days (response) until detection of the virus in the fish samples at the same farm site. Four sites (S_A, S_C, S_E, and S_F) were positive for SAV in both the seawater- and fish samples, by reverse transcription quantitative real-time PCR (RT-qPCR). Both the responses and the predictors are back-transformed from the log scale. The relationship between the number of SAV copies and the number of days until detection in fish samples at each of these sites was negative, meaning that the more SAV copies found in the seawater, the closer in time was SAV detection in fish. The vertical black line illustrates the limit of quantification (LOQ: 3.44 $\log_{10}$ [copy number L^{-1}]), when using the filtration method and considering the 25 % recovery, as calculated by Bernhardt et al. (2021).

12. Appendix

This registration form was made for the fish health inspectors to be filled out with every shipment of water samples that had been collected at the marine farm sites during the main field study (**Paper III**).

Name of the person who collected the seawater samples	First name: Last name:			Date:	Location:		
Farm site ID number							
What fish species are at the site? (mark with X the correct option)	□ Atlantic salmon (*Salmo salar* L.) □ Other (describe)...						
SEAWATER SAMPLES Fill in the detailed description for each individual net-pen from which seawater samples were collected. Also state the reasons if seawater sample(s) were not collected from the net-pen(s).	Name on bottle	Net-pen number	Date for seawater sampling (day/month/year)	Time for seawater sampling	Number of bottles (1 bottle à 1 L)		Number of dead fish for monthly analysis
	A						Dead: Euthanized: Total:
	B						Dead: Euthanized: Total:
	C						Dead: Euthanized: Total:
	D						Dead: Euthanized: Total:
	E						Dead: Euthanized: Total:
	F						Dead: Euthanized: Total:
	G						Dead: Euthanized: Total:
	H						Dead: Euthanized: Total:
	I						Dead: Euthanized: Total:
	J						Dead: Euthanized: Total:
SEAWATER PARAMETERS Should be measured just below the seawater surface. (Noted values for the following parameters must be measured on the same day as the seawater samples were collected)	Temperature (°C)	Salinity (‰)	pH	Oxygen level(O_2)			
OTHER COMMENTS For example: Deviations, observations (e.g. abnormal swimming pattern, visible fins on the water surface, loss of appetite ... etc), bad weather conditions, issues during sampling etc.							

Errata.list:			
Page	**Line**	**Change from**	**Change to**
Front page	English and the Norwegian titles	Font size 12	Correction: To font size 16 and bold.
		salmonid alphavirus	salmonid alphavirus
	All text	Line spacing 1.1. Font: Times New Roman	Line spacing 1.5. Font: Arial
All text	Titles Body text	Times New Roman Times New Roman	Arial Cambria
Table of contents	Appendix	With regards to: Page number 73	(Correction: Removing page number "73" in table of contents since Appendix is given in the end of the thesis, after the scientific papers and therefore no page number is needed)
1	11	show	shows
2	4	the oil and gas industry, which is the most important seabased exporter (Johansen et al. 2019).	the oil and gas industry (Johansen et al. 2019).
2	23	starts	start
2	28	containing	contain
4	31	from early harvest,	during
5	8	and typical initiatives for dealing with each are summarized	are presented
6	32	Pancreas	pancreas
7	25	(PMV)	(PMCV)
7	18	of high	of the high
8	2	of infection	of the virus
8	29	as reported, e.g.	as reported for, e.g.
9	4-5	every second virus cause over 1000 virus infections in the world's oceans (Suttle 2007).	every second, virus cause approximately 10^{23} virus infections in the world's ocean (Suttle 2007).

9	26	with an icosahedral symmetrical and spherical shaped genome	with an icosahedral single nucleocapsid that encloses a spherical shaped genome
9	32	(**Figure 4**)	(**Figure 3**)
10	2	**Figure 4.**	**Figure 3.**
10	14	(**Figure 5**).	(**Figure 4**).
10	31	**Figure 5.**	**Figure 4.**
11	23	by a another	by another
12	8	**Figure 6.**	**Figure 5.**
12	14	***Figure 6.***	***Figure 5.***
13	17	(**Figure 3**).	(**Figure 6**).
13	19	(Ferguson et al. 1986b a)	(Ferguson et al. 1986a,b)
14	2	***Figure 3.***	***Figure 6.***
14	1/Image	The image is unclear in the small size given	(Correction: Enlargement of the image could clarify the details)
14	21	well boats	well-boats
16	26-27	(Jansen et al. 2010a b)	(Jansen et al. 2010a,b)
17	20	Clinical signs and gross	Clinical signs and gross (Correction: Added space before "Clinical", since it's a new section)
18	2	SAV/PD to differentiate between	SAV/PD, to differentiate between
18	Table 2	skeletal) muscle	skeletal muscle
18	Table 2	Heart and mid-kidney.	Heart and mid-kidney
18	6	BF-2 = Blue Fin-2	BF-2 = Bluegill Fry-2
23	3	and prevent	and to prevent
25	24	Atlantic salmon (*Salmo salar* L.)	Atlantic salmon
31	Table 4	IPC	internal process control (IPC)
32	8	Cohabitant	The cohabitant
32	26	15.5 kg/m^3	15.5 kg m^{-3}
33	6	~ 15 cm	~ 0.15 m
33	15-16	temperature	water temperature

35	7-8	from, occasional	from, and sometimes occasional
35	10	For this reason	For these reasons
37	23-24	by a pH-adjusted solution is	by a pH-adjusted solution
37	32	In this study,	In the present study,
38	18	In general	In general (Correction: Made it into a new section)
40	9	virus pathogen	virus
39	28-29	extracted tissue	extracted fish tissue
39	32-33	a challenge	the challenge
41	21	inhibitors, can reduce	inhibitors, which can reduce
42	4	(Andersen et al. 2010, Jarungsriapisit et al. 2016a,b, 2020, Paper II).	(Andersen et al. 2010, Jarungsriapisit et al. 2016a,b, 2020).
42	32	and to detect viruses in water samples	and in water samples
45	4-6	the ICH Q2 (R1) guideline	the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) Q2 (R1) guideline
45	13-14	from the highest dilution giving results with a low giving results with a	from the highest dilution giving results with a low (Correction: Removed repetition of the same wording)
45	27	, variability	, variable and
48	1	This MF-/lysis	The MF-/lysis
49	10	the LOD was found	the LOD and LOQ were found
50	5	concentrate to SAV	concentrate SAV
50	7-8	The filter-buffer	In the present study, the filter-buffer
50	14-15	fish population. Furthermore,	fish population. Furthermore, (Correction: Chunky paragraph. Hence, “Furthermore” has become a new section)
51	3	In our study,	In our study, (Correction: Chunky paragraph. Hence, “In our study” became a

51	7	This is highly related	new section together with "This is highly related..:")
52	14	Water surveillance	Water surveillance (Correction: No need for space before the word "Water…")
52	21	water environmental	water for environmental
54	5	, negative	, a negative
54	12	analysis consistently	analysis was consistently
54	28	was pure	were pure
59	3	The new filtration method successfully detected SAV from seawater even at the earlier	The new filtration method successfully detected SAV from seawater even at the earlier (Correction: No need for space before the word "The new…")
66	Reference	Hodneland K, Bratland A, Christie K, Endresen C, Nylund A (2005a) New subtype of salmonid alphavirus (SAV), Togaviridae, from Atlantic salmon Salmo salar and rainbow trout Oncorhynchus mykiss in Norway. Dis Aquat Organ 66:113–120.	(Correction: Removing this reference since the same reference was listed in the reference list)
69	Reference	Lillehaug A, Santi N, Østvik A (2015) Practical Biosecurity in Atlantic Salmon Production. J Appl Aquac.	Lillehaug A, Santi N, Østvik A (2015) Practical Biosecurity in Atlantic Salmon Production. J Appl Aquac 27:249–262.
72	References	Segner H, Reiser S, Ruane N, Rösch R, Steinhagen D, Vehanen T (2019) Welfare of fishes in aquaculture. Budapest.	Segner, H., Reiser S, Ruane N, Rösch R, Steinhagen D, Vehanen T (2019) Welfare of fishes in aquaculture. FAO Fisheries and Aquaculture Circular No. 1189. Budapest.
74	References	Thodesen J, Gjedrem T (2006) Breeding programs on Atlantic salmon in Norway: lessons learned.	Thodesen, J. and Gjedrem, T. (2006) 'Breeding programs on Atlantic salmon in Norway: lessons learned', Development of Aquatic Animal Genetic Improvement and Dissemination Programs: Current Status and Action Plans, pp. 22–26.

Paper 3 page 9	15-16	$N_1 = N_2 \cdot (1+E)^{(Cq_1 - Cq_2)}$	$N_1 = N_2 \cdot (1+E)^{(Cq_2 - Cq_1)}$
Paper 3 page 9	17	Inter-plate calibrator	IPC
Paper 3 page 9	18	the calibrator, respectively. The calibrator	the IPC, respectively. The IPC
Paper 3 page 12	20-23	and two SAV-positive samples at 0.15 m with estimated copy numbers below LOD.	(Correction: this was not correct according to the results given and was therefore removed from the text)
Paper 3 page 14	21-22	by RT-qPCR. At S_D, S_F and S_G	by RT-qPCR. At S_A, S_D, S_F and S_G
Paper 3 page 14	23	The seawater	The seawater (Correction: Made it into a new section)
Paper 3 page 18	2	desireable	desirable
Paper 3 page 24	Table 1	With regards to: “SAV-positive samples are high lightened in green”	(Correction: SAV-positive samples have been high lightened in green in Table 1)

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