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

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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 at 7 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 these 7 sites. The samples were 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, at all sites where the fish tested positive for SAV. A significant negative 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 the SAV particles in the seawater, the more days it took until SAV was detected in the 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.

KEY WORDS: 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, for example, 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 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, and it is the aetiological agent of pancreas disease (PD) (Weston et al. 2002, Hodneland & Endresen 2006).

The first report of 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 7 subtypes of SAV (SAV1–SAV7) are known (Fringuelli et al. 2008, Tighe et al.

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2020). Two of these are known to be present in Norwegian aquaculture facilities, i.e. SAV2 and SAV3, forming 2 separate PD endemic zones with marine SAV2 on 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 the whole of Norway and 2 national surveillance zones for SAV2 and SAV3 (Lovdata 2017). This program requires monthly sampling of fish from marine farm sites with Atlantic salmon, rainbow trout *Oncorhynchus mykiss* 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. Testing is conducted on all active marine farm sites until positive detection of SAV in fish samples. 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 to be representative of 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. In Norway, detection of SAV can lead to measures and/or restrictions, meaning the whole fish population at the site is allowed continued growth until slaughtering. However, detection of SAV outside the endemic zone can lead to slaughtering of the whole fish population at the site.

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). Therefore, we 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, compared to detection from sampling of limited fish numbers. 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 of 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 at 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 3 mo earlier under the surveillance program. This site had 5 floating open marine net-pens with Atlantic salmon, which had been sea transferred in August and September 2017, in Hordaland county (note that Hordaland, Sogn and Fjordane counties were merged into the new Vestland county on 1 January 2020) in Western Norway (inside the SAV3 endemic zone).

In this study, seawater sampling was carried out from one net-pen (∅ 50 m) enclosing ~180 000 Atlantic salmon with an average weight of ~2.5 kg. One litre seawater samples were collected in duplicate from 3 sampling depths (0.15, 5, and 10 m) and at 3 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) by hand down to a depth of 0.15 m, allowing the water to fill the plastic bottle. For the other 2 sampling depths (5 and 10 m), a Ruttner 2 l Standard Water Sampler (Hydro-Bios) was used. The filtration of the seawater

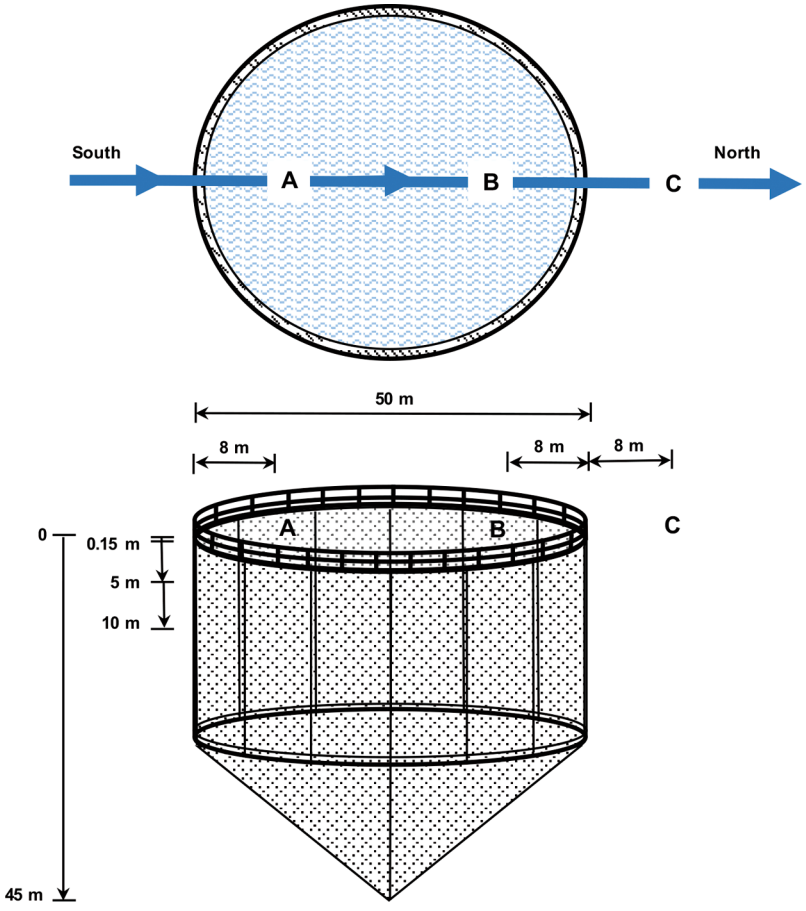

Fig. 1. Schematic illustration of the sampling design for the pilot study on detection of salmonid alphavirus (SAV) in seawater from a farm holding pancreas disease diagnosed fish. Duplicate 1 l samples were collected from 3 different depths (0.15, 5, and 10 m) at 3 different sampling points (A, B, and C) in relation to the 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

samples took place on site. Seawater temperature (°C) was measured at the 3 sampling depths on the day of sampling 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 9 mo, 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 at 5 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 2 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 route by water between the sites, around islands, peninsulas or other hindrances) of ~3 and ~2.7 km, respectively. S_F and S_G are situated with a sea-way distance of ~10.7 km. S_A was always sampled independently due to its distant location in relation to the other sites, whereas 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 the 7 sites at 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, a 1 l seawater sample was collected from every net-pen at each of the 7 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 7 sites. One litre of seawater was collected monthly inside each net-pen at a site, using a 1 l sterilized plastic bottle (VWR), which was vertically positioned in a bottle holder, attached to a 65–120 cm telescopic swing sampler (Bürkle). The bottle was swung horizontally while submerged approximately 0.15 m below the water surface, close to the net-pen's inner side. In addition, 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).

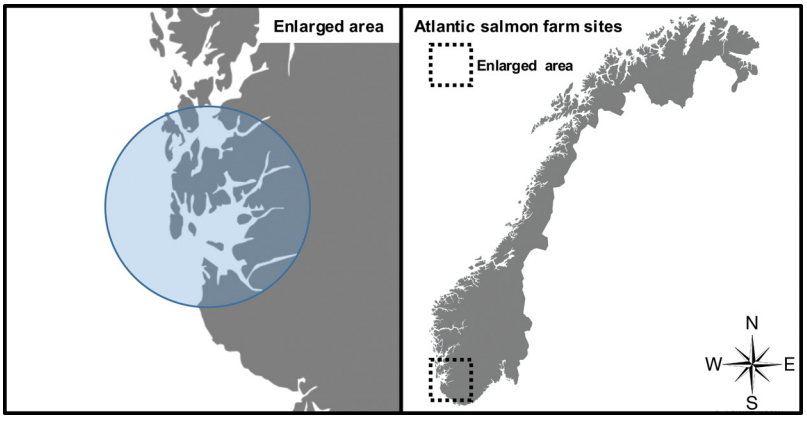

Fig. 2. Location of the 7 Norwegian marine farm sites (S_A–S_G) of Atlantic salmon *Salmo salar* that were sampled for seawater during May 2019–January 2020. (Edit of the original map, retrieved from https://www.vemaps.com)

Following sampling, all bottles from each 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). The samples were sent to the laboratory of the Norwegian Veterinary Institute (NVI, Oslo, Norway) by express delivery (within 24 h), measured for 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, such as transfer of the fish populations between net-pens on the location due to, for example, treatment). The seawater temperature (°C) for each site was measured at 3 m depth in the same week as the seawater sampling took place (data retrieved from www.barentswatch.no/fiskehelse/).

2.3. Concentration of seawater samples

The concentration of 1 l seawater samples was measured as previously described (Weli et al. 2021). Filtration was performed using a 47 mm electronegative charged membrane filter (MF-Millipore 0.45 µm MCE membrane, Merck Millipore) into a 47 mm in-line filter holder (Merck Millipore). The 1 l seawater samples were filtered using a peristaltic pump (V6-3L Peristaltic Pump, Shenchen) 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) 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 (~5.5 × 10^3 PCR units) 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 using the easyMAG robot (bioMérieux) and the standard lysis protocol (generic 2.0.1.) with 50 µl magnetic silica beads, according to the NucliSENS easyMAG user manual (https://www.manualslib.com/manual/1377074/Biomerieux-Nuclisens-Easymag.html). 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 studies was made by RT-qPCR, as previously described (Weli et al. 2021). A broad-spectrum Q_nsP1 assay was used, targeting the nsP1-gene in SAV, giving amplicons of 107 bp (Hodneland & Endresen 2006). Each primer had a final concentration of 500 nM (R-primer: 5′-GTA GCC AAG TGG GAG AAA GCT-3′; F-primer: 5′-CCG GCC CTG AAC CAG TT-3′) and a final concentration of 300 nM of the probe (FAM-5′-CTG GCC ACC ACT TCG A-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 RT-qPCR kit used was TaqMan Fast Virus 1-Step Master Mix (Applied Biosystems), 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 and 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 triplicate, while samples from the pilot study were run in duplicate, by RT-qPCR. In cases of some parallels giving a negative result, samples were rerun in triplicate before being assigned negative or positive (a total number of 3–5 positive replicates defined a positive sample).

2.6. Quantification of SAV copies

Quantification of SAV was done 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 is the amplification efficiency, and Cq_1 and Cq_2 are the quantification cycle (Cq) values for the sample and 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 2-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 fulfill 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

$$20 \times 2.4 \times \frac{1}{R}$$

where R is the recovery approximated to be 0.25 (25%), as calculated according to Weli et al. (2021). The SAV data were $\log_{10}$ transformed before being plotted using Excel (Microsoft Office Professional Plus 2016).

The mengovirus RNA 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 PD (i.e. ≥20 fish per site) at all 7 sites. Samples of the spongious tissue from the cardiac ventricle were stored in RNAlater, processed and analyzed for SAV by RT-qPCR at 3 different 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 subjected to Sanger sequencing of the SAV E2 gene (which allows separation between all known SAV subtypes), on an Applied Biosystems 3500xl Genetic Analyzer with BigDye Terminator v3.1 Cycle Sequencing Kit, according to standard protocols at NVI. An additional PCR for the SAV E2 region was performed, and primers identical to those previously used in the PCR amplification step were used for sequencing (F-primer: 5′-GCC ACC ACC TGT CCG ATC TG-3′; R-primer: 5′-ACC AAG GTT CCG TGT AGT TAG C-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 relationship between the number of SAV copies detected in seawater samples and the number of days between collecting these samples and detection of SAV in heart tissue from fish. The number of days is bound by the generations at the sites that were monitored. Therefore, if SAV was not detected in fish tissue samples at a site during one generation, this may be because the fish population had not been infected, or because it was slaughtered before the viral load was detectable. In the latter case, the number of days is unknown. These so-called 'right-censored' data are common in survival analyses. A survival regression was employed under the assumption that the errors followed a Weibull distribution using the 'survreg' function in the 'survival' extension package in R (Therneau & Grambsch 2000, Therneau 2015). In this case, the 'survival' is synonymous with keeping the status as 'not infected' from fish samples. More information

about Weibull regression models in R can be found in Zhang (2016).

In addition, the number of SAV copies recovered using RT-qPCR has a limit of detection (LOD). If viral RNA was not detected in the sample, this may be because the virus was not present, or was present in concentrations below the LOD. In the latter case, the data were 'left-censored', which can be an issue with a large amount of non-detects. To overcome this issue, data below LOD were imputed as random draws from a log-normal distribution, where the mean and standard deviation were derived from censored data using the Kaplan-Meier method (Canales et al. 2018, Lee 2020). The LOD used in our calculations is from the study of Bernhardt et al. (2021).

Aldrin et al. (2015) have shown that stocking season, salmon abundance, salmon weight, and water temperature affect the SAV infection dynamics, and these factors may therefore also affect the number of days between a positive detection in water samples and a positive detection in heart tissue. The NVI has access to monthly salmon abundances, salmon weights, and production histories in Norwegian Atlantic salmon farms, through the extensive monitoring of the aquaculture industry, administered by the Directorate of Fisheries. These factors were therefore regarded as potential explanatory variables that could be tested statistically.

A possible relationship between SAV copies l^{-1} and days between detection in water and detection in fish may differ between the sites, due to differing oceanographic properties or other unmeasured effects. We therefore regarded site as a potential explanatory variable. In addition, given that positive water samples reflect the presence of virus shedding in the farm, we assume that the virus was present for the remainder of the production cycle. In some cases, SAV was detected on more than one sampling occasion at a site prior to the detection of SAV in fish tissue. To account for both of these issues, we calculated the cumulative average number of SAV copies l^{-1} in consecutive water samples at each site, and regarded this measure as a potential explanatory variable. In addition, the probability that a cohort becomes infected with SAV is expected to increase with time per se, as a monthly background infection rate. Hence, the following variables were regarded as possible explanatory variables prior to the model selection: stocking season, salmon abundance (log transformed), salmon weight (log transformed), age (number of months since stocking), water temperature, site (S_A–S_G), SAV copies l^{-1} in water samples, and the cumulative average of the latter. The number of SAV copies l^{-1} in water samples was tested with and without log transformation. All log transformations were employed using the natural logarithm, and 1 was added to the original value to avoid taking the logarithm of zero.

Samples were collected from all net-pens at each site and considered as pseudo-replicates. Therefore, the mean SAV copies l^{-1} from each site sampled at the same time point was included in the analyses. We employed a forward model selection using the Akaike's information criterion (AIC) as an optimization criterion, and competing models were tested using ANOVA 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). Models were plotted using the ggplot2 package in R (Wickham 2016).

3. RESULTS

3.1. Pilot study

A total of 18 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 3 water depths and sampling points differed, but showed the same trend at sampling points A and B (Fig. 3).

Inside the net-pen (i.e. sampling points A and B), the SAV concentrations decreased with water depth. 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 $\log_{10}$ (SAV copies l^{-1}) and 5.97 $\log_{10}$ (SAV copies 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), SAV detection revealed the opposite trend to sampling points A and B, with the highest SAV concentration at 10 m (5.08 $\log_{10}$ [SAV copies l^{-1}]) and the lowest SAV concentration (3.29 $\log_{10}$ [SAV copies l^{-1}]) at 0.15 m.

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

3.2. Field study

A total of 286 seawater samples were collected from all net-pens at 7 different Norwegian marine farm

sites of Atlantic salmon (S_A–S_G) during May 2019–January 2020 (Table 1). Additionally, 7 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 and 18.8°C (7.0 ± 3.4°C) upon arrival at NVI. Forty-two samples (15%) were SAV-positive, of which 25 (60%) showed an inhibitory effect. The results for each site are shown in Table 1.

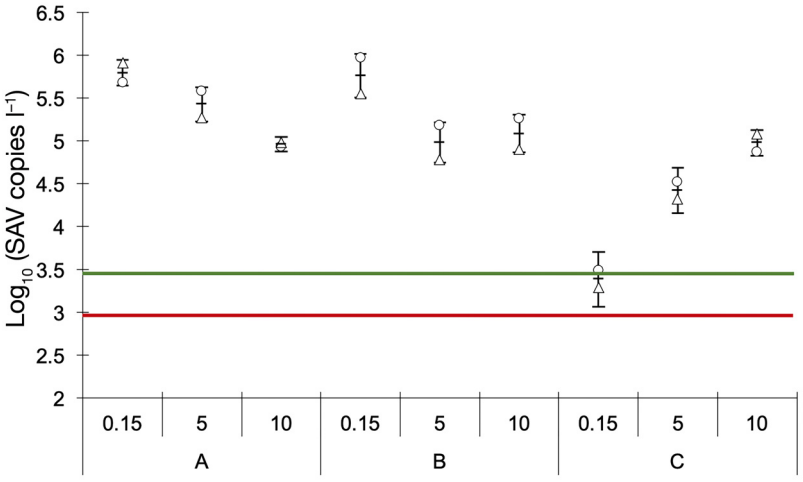

Fig. 3. SAV copies l^{-1} seawater from a net-pen holding pancreas disease diagnosed fish (pilot study). Quantification was performed using RT-qPCR. Nine seawater samples were collected in duplicate (2 × 1 l) from 3 sampling points (A, B, and C) at 3 depths (0.15, 5, and 10 m) (see Fig. 1). The SAV concentration in replicates 1 (circles) and 2 (triangles) is shown together with the mean (central bar) and SD (error bars). The limit of detection (LOD: 2.94 $\log_{10}$ [SAV copies l^{-1}]) and the limit of quantification (LOQ: 3.44 $\log_{10}$ [SAV copies l^{-1}]), as calculated by Bernhardt et al. (2021), are represented by the solid red and green horizontal lines, respectively

At S_A, SAV was first detected in the seawater in June 2019 from 1 of the 6 net-pens, i.e. 4 mo 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 until 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 4 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 $\log_{10}$ [SAV copies l^{-1}]) was found in the seawater sample collected from 1 of the 4 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 6. Of these, the fish populations in 2 net-pens were slaughtered in August and January, respectively, before water sampling, leaving only 2 net-pens for collection of seawater samples in the last month of the field study.

Low concentrations of SAV were found in seawater from only 2 out of 5 net-pens at S_B in late August (i.e. second sampling that month), and in October from 1 out of the 4 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 1 out of 5 net-pens in October. At S_E, SAV was detected in the seawater in 1 out of 10 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 1 out of 6 net-pens, then in all 5 net-pens in November, and then in 1 out of 2 and in 2 out of 2 net-pens in December and January, respectively. The highest concentration of SAV at S_F was found in seawater collected in November, and SAV was first found in fish samples in January. At S_G, SAV was detected in the seawater samples for the first time in August in 1 out of 7 net-pens, and next in January in 2 out of the 5 remaining net-pens, which tested 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.

A single 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 the 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 and 17.2°C (11.6 ± 2.9°C) at S_A, between 8.0 and 17.7°C (13.0 ± 3.4°C) at S_B, between 8.0 and 16.7°C

Table 1. Overview of salmonid alphavirus (SAV) detection by RT-qPCR in 1 l seawater and in fish collected on the same days during May 2019–January 2020, in connection with the national surveillance program for SAV/pancreas disease (PD). Samples were collected from all net-pens at 7 different sites (S_A–S_G) of Atlantic salmon *Salmo salar* that were sea transferred either in autumn 2018 (A-2018) or spring 2019 (S-2019). SAV-positive samples are marked in **bold**. For seawater, the number of SAV-positive samples out of the total number of samples is given (NB some months had 2 sampling days), and the SAV concentrations (log_{10} [SAV copies l^{-1}]) are shown within brackets. The limit of quantification (LOQ) is 3.44 log_{10} [SAV copies l^{-1}] for the RT-qPCR when using the filtration method, as calculated by Bernhardt et al. (2021). For fish, heart samples that tested positive and negative for SAV 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 fallowing. nd: no data

Site	Sea transfer	Type of sample	May 2019	Jun 2019	Jul 2019	Aug 2019	Sep 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	**2/5**	0/5	0/5	0/5	0/5	0/5	Jul 2020	None
		Fish	–	–	–	–	–	–	–	–	–		
S_C	S-2019	Seawater	nd	0/4	0/4	0/4	nd	**1/4**	0/4	0/4	0/4	Jun 2020	Jun 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**[a]	0/10	**2/10**	0/10	**5/10**	nd	nd	nd	Mar 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	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	0/7, 0/6	0/5	nd	**2/5**	Feb 2020	None
		Fish	–	–	–	–	–	–	–	–	–		

[a]9 of 10 seawater samples were analyzed

(11.5 ± 2.9°C) at S_C, between 11.9 and 18.4°C (14.7 ± 2.9°C) at S_D, between 11.6 and 18.2°C (14.4 ± 2.9°C) at S_E, between 6.5 and 19.0°C (12.7 ± 4.1°C) at S_F, and between 6.7 and 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 2 sites (i.e. S_F: $Cq > 30$ and S_G: $Cq > 35$) did not provide 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 4 out of the 7 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. The final model included site (S_A–S_G), fish age, log-transformed cumulative average of SAV copies l^{-1}, and salmon abundance (log transformed). The linear predictors from this model are presented in Table 2 and Fig. 4 to illustrate the functional relationship between number of days between seawater sampling and SAV detection in fish. All combinations of explanatory variables that were tested during model selection gave the same qualitative result: SAV copies l^{-1} was a highly significant predictor of the number of days between SAV detection in seawater and in fish samples at the same site, with a negative estimate. Thus, there was a significant negative relationship between the cumulative average SAV copies l^{-1} and number of days between SAV detection in seawater and in fish samples at the same site (Fig. 4, Table 2). This means that with a higher

Table 2. The model selection revealed that the number of days between SAV detection in seawater and in fish was an effect of the farm site and the cumulative average SAV copies in 1 l seawater. Therefore, the final model included days between detections as a response variable, while the cumulative average SAV copies l^{-1}, salmon abundance, age (months since stocking), and site are the predictors. The estimate for Site A (S_A) is the intercept, while the other sites are presented as differences from the intercept (contrasts). Shaded rows illustrate arbitrary contrasts estimated for the sites that had samples that were all negative

	Estimate	SE	p
Intercept — Site A (S_A)	5.38	0.58	<0.001
Site B (S_B)	6.87	0.00	<0.001
Site C (S_C)	0.80	0.28	0.044
Site D (S_D)	269.32	0.00	<0.001
Site E (S_E)	1.76	0.16	<0.001
Site F (S_F)	1.83	0.13	<0.001
Site G (S_G)	1281.06	0.00	<0.001
Cumulative average of copies l^{-1} (log)	−0.65	0.047	<0.001
Salmon abundance (log)	0.20	0.024	<0.001
Age	−0.084	0.037	0.022
log(scale)	−1.47	0.16	<0.001

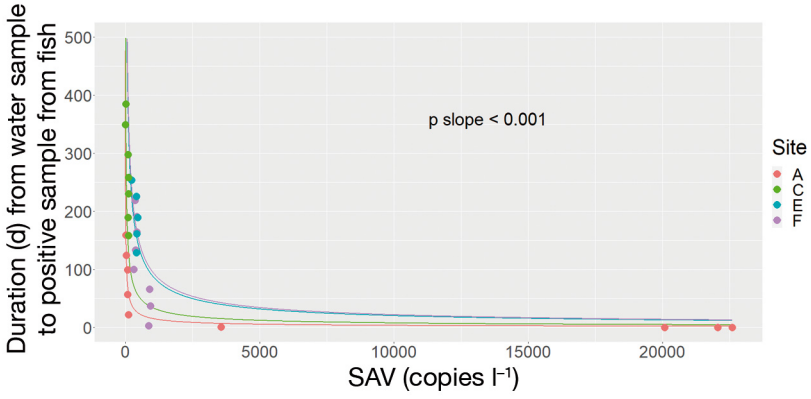

Fig. 4. Relationship between the cumulative average number of SAV copies (predictor) in 1 l seawater and the number of days (response) until detection of the virus in the fish samples at the same farm site. SAV copies were quantified by RT-qPCR. Four sites (S_A, S_C, S_E, and S_F) were positive for SAV in both the seawater and fish samples. Both the response and the predictors are back-transformed from the log scale. The relationship between SAV copies l^{-1} 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

SAV copies l^{-1}, the SAV detection in fish occurred closer in time.

SAV copies were quantifiable only at S_A and S_F, and much of the trend in the model was driven by the data from S_A. Two new reduced models were therefore fitted as a sensitivity analysis, using only S_A data and using all data omitting S_A data. The reduced models both produced a significant reduction in the expected number of days to SAV detection in fish tissue with increasing virus concentrations in water samples (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 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 the subsequent large-scale field study. Seawater samples collected from the top layer (0.15 m) and greater depths (5 and 10 m) showed up to a 10-fold difference in SAV concentrations, with the highest concentrations from the top layer. Additionally, sampling from the top layer constituted by far the most practical method for application under field conditions, and gave support to the choice of the sampling procedure for the large-scale field study.

In the large-scale field study, we compared surveillance of SAV at marine farm sites of Atlantic salmon using sampling of seawater versus screening of fish. Results showed that detection of SAV was made earlier in seawater than in fish samples at 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 SAV status and allowed an earlier detection of the virus, versus sampling of relatively few fish individuals.

At sites with positive SAV detection in the water samples, but not in the fish samples (i.e. S_B, S_D and S_G), this might be explained by having too few infected fish to be detected in a limited fish sample (i.e. small prevalence of infected fish). However, if the fish had been monitored for a longer period of time at these sites, a higher prevalence could have been

found, allowing SAV detection in the fish during the fish screening. In contrast, if the environmental conditions did not facilitate an escalation of the prevalence at these sites, fish screening might have stayed negative, which highlights the role of the 'epidemiological triad' (Snieszko 1974).

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 the LOD, as calculated by Bernhardt et al. (2021) (Fig. 4). This 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. Specifically, the actual recovery of the virus will vary from sample to sample, and the SAV recovery in our samples was probably higher than the 25% as calculated in the *in vitro* study (Weli et al. 2021) and used in our LOD calculation. This means that our LOD is a 'conservative' estimate, which does not overestimate the sensitivity of the method. The LOD and the limit of quantification (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 the water temperature at the time of infection may be connected to the pathogenesis of PD; when the water temperatures are rising, more acute and shorter-lived outbreaks occur, whereas when water temperatures are decreasing, 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 in colder 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, and was carried out according to the regulations; as such, the sampling was 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 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 the large number of seawater samples in the present study, RT-qPCR was used for detection of 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, the present 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 NVI laboratory 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. Alternatively, the filter could be stored in some form of transport medium (i.e. lysis buffer). Overall, there is a need for optimization of the method and screening design by carrying out 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 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, 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, as sampling of fish would probably be unnecessary in periods with SAV-negative water samples. SAV detection in water, which indicates a significant likelihood of subsequent SAV detection in fish, could also initiate implementation of disease control measures on neighbouring farms, allowing improved biosecurity measures and more effective health management of marine-farmed Atlantic salmon. The water analysis may also have the potential for surveillance of other pathogens in farmed fish populations. However, further optimization, validation, and practical use of this new water filtration method, alongside screening of fish, are required to evaluate the implementation of water filtration for surveillance of SAV in a fish population.

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