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The effect of temperature on the survival of salmonid alphavirus analysed using *in vitro* and *in vivo* methods

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ABSTRACT

Disease outbreaks in fish aquaculture are often of concern due to the possibility of pathogen transmission to fish in neighbouring farms and to wild fish populations. To be able to assess the risk and manage transmission and outbreak scenarios, it is of great importance to understand the impact of various biological and physical conditions affecting the survival of the pathogen in the environment. In this study, we examined the effect of temperature on the viability of salmonid alphavirus subtype 3 (SAV3) in three separate experiments using SAV3 in culture medium, SAV3 in seawater or SAV3 shed by infected fish mimicking the virus produced at marine sites during outbreaks. SAV cultured on CHH-1 cells and cultured SAV mixed with seawater were incubated at different temperatures over a period of 4 weeks and 2 weeks, respectively. The surviving virus was quantified by two *in vitro* methods, RT-qPCR and a 50% tissue culture infectious dose (TCID50) assay. In addition, seawater containing SAV shed by Atlantic salmon post-smolts was incubated at different temperatures over 4 weeks. The viability of SAV shed by SAV-infected fish was examined both by *in vitro* methods and assayed *in vivo* using recently seawater-transferred post-smolts. The survival of SAV decreased with increasing temperature in all three experiments. Generally, the titre of SAV propagated in cell culture was more stable in culture medium compared to SAV diluted in seawater or shed by infected fish. Cells could be infected with a low titre of SAV, 24 TCID50 L^{-1} of seawater, whereas a SAV concentration above 448 $TCID_{50}$ L^{-1} of seawater was required for successful SAV transmission to the post-smolts in the *in vivo* assay. SAV3 shed by infected fish at a starting titre of 882 $TCID_{50}$ L^{-1} of seawater was completely inactivated after three and four weeks of incubation at 16 °C and 12 °C, respectively, as analysed by the TCID50 *in vitro* assay. In addition, the TCID50 resulted in a higher sensitivity than RT-qPCR, especially at very low virus titres, probably due to the multiplication of the virus in cells and the possibility of analysing a greater volume of sample in the assay compared to RT-qPCR. The information acquired in the present study may be incorporated into pathogen dispersal models in order to aid understanding and management of SAV outbreaks. Calculation of the risk of transmission would provide new prospects for the control of pancreas disease.

1. Introduction

Aquatic animals including finfish, molluscs, and crustaceans are important protein sources and accounted for 17 percent of global animal protein supply in 2012 (FAO, 2016; The World Bank, 2013). In addition, the annual growth rate of the world fish production from aquaculture during 2000–2010 was 7.2 percent which is higher than from meat (2.6%), and egg, milk and processed milk production (2.5%) (FAO, 2013). This indicates a potential for further aquaculture growth. Viral infections are global problems in aquaculture and are negatively affecting the expansion and development of the aquaculture industry.

In Norway, the major species in aquaculture is Atlantic salmon, reaching 1.2 million tonnes of slaughtered fish for food and an estimated 61.6 billion NOK in sales value in 2017 (Statistics Norway, 2018). In 2017, the top three viral diseases affecting farmed salmonids in Norway were skeletal muscle inflammation (HSMI) caused by piscine

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Abbreviations

CPE	cytopathic effect
L-15	Leibovitz-15 cell culture medium
PD	pancreas disease
RT-qPCR	quantitative reverse transcription polymerase chain reaction
SAV3	salmonid alphavirus subtype 3
TCID50	50% tissue culture infectious dose

reovirus (PRV), cardiomyopathy syndrome (CMS) caused by piscine myocarditis virus (PMCV) and pancreas disease (PD) caused by salmonid alphavirus (SAV) (Norwegian Veterinary Institute, 2018). Infection of Atlantic salmon by SAV causes pancreas disease and contributes to the substantial mortality during the seawater phase of salmonid aquaculture in Europe. Mortalities associated with PD are not the only cause of financial loss, weight reduction and down-grading of fillet quality at slaughter also contribute to the losses incurred (Larsson et al., 2012; Taksdal et al., 2015).

SAV is an enveloped virus with a single stranded positive-sense RNA genome. There are 6 subtypes identified based on variations in their nsP3 and E2 genes (Fringuelli et al., 2008). These subtypes are geographically distributed in northern Europe in Atlantic salmon farming areas. Along the Norwegian coastline SAV 2 and 3 have been detected (Hjortaas et al., 2013, 2016). SAV3 has been reported to cause higher mortalities and more severe symptoms than SAV2 (Jansen et al., 2015; Taksdal et al., 2015).

PD is a notifiable disease in Norway, therefore PD outbreaks and the detection of SAV at farmed sites must be reported and management regulations introduced (as cited in Bang et al., 2012). Pathogen transmission in the field is complex since many of the factors such as shedding rate, the exact time of infection, dilution factor, wind strength, and water currents are difficult to measure and control. There is a potential for transmission of pathogens from sea cages holding diseased fish to surrounding waters and to neighbouring and/or distant sites. While disinfection is a highly effective preventive measure for land-based production, it is impossible to practise in the borderless marine environment. Vaccination is an alternative measure for disease control in the open marine environments. However, SAV continues to be detected along the Norwegian coast despite the use of vaccines by several producers indicating that vaccines against SAV are not completely effective. (Bang et al., 2012; Deperasińska et al., 2018). Thus, knowledge about how long viruses such as SAV can remain infective in their natural habitat provides a crucial understanding of transmission, epidemiology and disease management. Information about pathogen survival under specific environmental conditions combined with host susceptibility to infectious agents, viral shedding, and minimum infectious dose would allow improvement of predictive modelling when estimating the impact of disease outbreaks (Garver et al., 2013). Furthermore, it is useful for planning and implementing effective control measures.

Regarding SAV survival outside its host, the biophysical properties of SAV1 and SAV2 under different parameters, such as pH, temperature and presence of organic matter have been studied and it has been shown that extreme pH values, high temperatures and organic loads reduce the virus titre (Graham et al., 2007; Villoing et al., 2000). However, there is still a need for more data on the transmission of PD and survival of SAV. Although the genetic variations between the subtypes are small, differences in infectivity between isolates within the same subtype and between subtypes have been shown (Jansen et al., 2015; Taksdal et al., 2015). SAV3 is the major cause of PD in Norway, therefore, the survival and transmission of SAV3 is the focus of the present study. To date, SAV survival studies have used virus propagated in cell culture and measured its infectivity using cell culture techniques such as end-point titration (Graham et al., 2007; Villoing et al., 2000). Since SAV is an enveloped virus and envelope integrity is critical for survival, the source of envelope formation is of interest (Pinon et al., 2018), and it is plausible that differences in the survival between laboratory-propagated virus and virus shed by infected fish might exist, in part due to the origin of the membrane (Pinon et al., 2018).

There is still limited information about how long the virus survives in the environment and maintains its infectivity and how much virus is required to establish SAV infection in an Atlantic salmon. Seawater containing virus shed by infected fish should more closely represent virus shed by infected fish in sea cages. A previous study has demonstrated that exposure to doses as low as 7 and 48 TCID50 L^{-1} of SAV3 shed by shedder fish into seawater was able to induce SAV3 infection in challenged fish by bath immersion (Jarungsriapisit et al., 2016a; Moore et al., 2017). The aim of the present study was thus to examine the effect of temperature on the length of survival of SAV using *in vitro* cell culture to measure infectivity. A secondary aim was to see if there were any differences in survival due to the origin of the SAV; cultured SAV, cultured SAV in seawater, and virus shed from SAV-infected fish. Furthermore, it is important to check whether the virus that survives and can infect cells in cell culture is also capable of infecting fish. This information will be crucial for the management of SAV transmission at the marine sites. Thus, the infectivity of SAV shed from SAV-infected fish and exposed to various temperatures was investigated using a realistic *in vivo* experimental infection model (Jarungsriapisit et al., 2016b) in order to obtain practical information for application in risk assessment.

2. Material and methods

2.1. Seawater and fish husbandry

Throughout all the experiments the term seawater refers to the seawater supplied to the wet-lab facility at the Institute of Marine Research (IMR), Bergen, Norway. The water is pumped from a depth of 128 m in the adjacent fjord where the salinity is 34.5‰. Seawater is maintained in reservoir tanks for sedimentation, pumped up to a header tank and supplied to the animal facility through free fall. All incubations of seawater in this study were carried out in the dark, in either climate-controlled rooms (Exp. A and C) or using incubators (Exp. B) where the set-temperature varied ≤ 0.5 °C. For fish rearing (Exp. C – *in vivo*), seawater in the tanks was maintained at a constant temperature of 12 °C, 300 L h^{-1} flow rate and > 80% oxygen saturation. During a pilot examination of the seawater supplied to the facility, total carbon phosphate and nitrate altogether representing organic load was low giving an indication that this should not have any impact on the survival of SAV3 in this study. Intake seawater was tested and confirmed to be negative for SAV.

Altogether, 226 fish were used in Experiment C, of which 96 smolts were used in the *in vivo* exposure to the virus containing seawater, while the rest were used as shedders. Unvaccinated Atlantic salmon smolts (Aquagen strain cultured by Matre aquaculture station, IMR) weighing approximately 75 g were transported in freshwater to IMR, Bergen. The fish were tested and confirmed to be negative for any known infections including SAV. Fish were fed *ad libitum*, transferred to seawater and acclimatized for 5 days prior to the beginning of the experiment.

Fish were anaesthetized with a mixture of Metomidate (10 mg L^{-1}) and Benzocaine (60 mg $L^{-1)}$ before handling and euthanized with a mixture of Metomidate (10 mg L^{-1}) and Benzocaine (120 mg L^{-1}) before sampling.

This study was approved by the Norwegian Food Safety Authorities (ID: 10419) and carried out in strict accordance with the guidelines.

2.2. Virus propagation and viral analytical methods

SAV3 isolated from heart tissue of Atlantic salmon (Xu et al., 2010),

was supplied by Dr. Øystein Evensen, NMBU, Norway, and was further cultivated using Chum salmon heart-1 (CHH-1) cells. CHH-1 cells were cultivated in 75 cm^2 plastic cell culture flasks with Lebovitz-15 medium (L-15) (Life Technologies, UK) supplemented with 2% foetal bovine serum (FBS) at 15 °C for virus propagation. Virus was harvested at 7 days post-infection when cytopathic effect (CPE) was observed. A 50% tissue culture infectious dose (TCID50) titre of the virus stock was calculated using a TCID50 assay (Ramakrishnan, 2016).

In both Experiment B and C, the *in-vitro* analysis of virus in seawater was carried out as described here. The virus in the seawater was concentrated from duplicate samples using electropositive 1 MDS filters with a filtration speed of 50 mL min^{-1} as described previously (Jarungsriapisit et al., 2016a). After filtration, the filters were placed upside-down in separate petri-dishes containing 1.2 mL of L-15 supplemented with 10% FBS and eluted at 500 rpm for 15 min on an orbital shaker at room temperature. The eluants were passed through a 0.22 µm syringe filter unit (Merck Millipore, Germany). In this way the virus in the water was binding to the filter and concentrated. One hundred microlitres of each eluant were then transferred into a microtube containing 350 µL of lysis buffer and stored at −80 °C prior to RT-qPCR analysis. The remaining eluant was analysed to check infectivity on CHH-1 cells using the TCID50 assay.

2.3. Experimental designs

In the present study, three experiments were designed to determine SAV survival at different temperatures, namely Experiment A, B, and C (Fig. 1A and B). Due to the availability of facilities and personnel, Experiment A and C were carried out at the same time while Experiment B was conducted separately. Although temperatures used in the experiments were different due to technical practicalities, all are relevant temperatures with regards to sea temperatures in Norway. In Experiment A, SAV cultured on CHH-1 cells was incubated at three different temperatures and the samples were analysed for SAV survival using a TCID50 assay on CHH-1 cells (Ramakrishnan, 2016) over a period of 4 weeks. In Experiment B, cell culture propagated virus was mixed with seawater and incubated at different temperatures and analysed for SAV survival using CHH-1 cells over a period of 2 weeks. In Experiment C, cultured virus (the same virus used in Experiment A) was injected into Atlantic salmon post-smolts in order to induce viraemia and cause shedding of infectious virus into the seawater in the tank. This seawater containing SAV was collected and incubated at three different

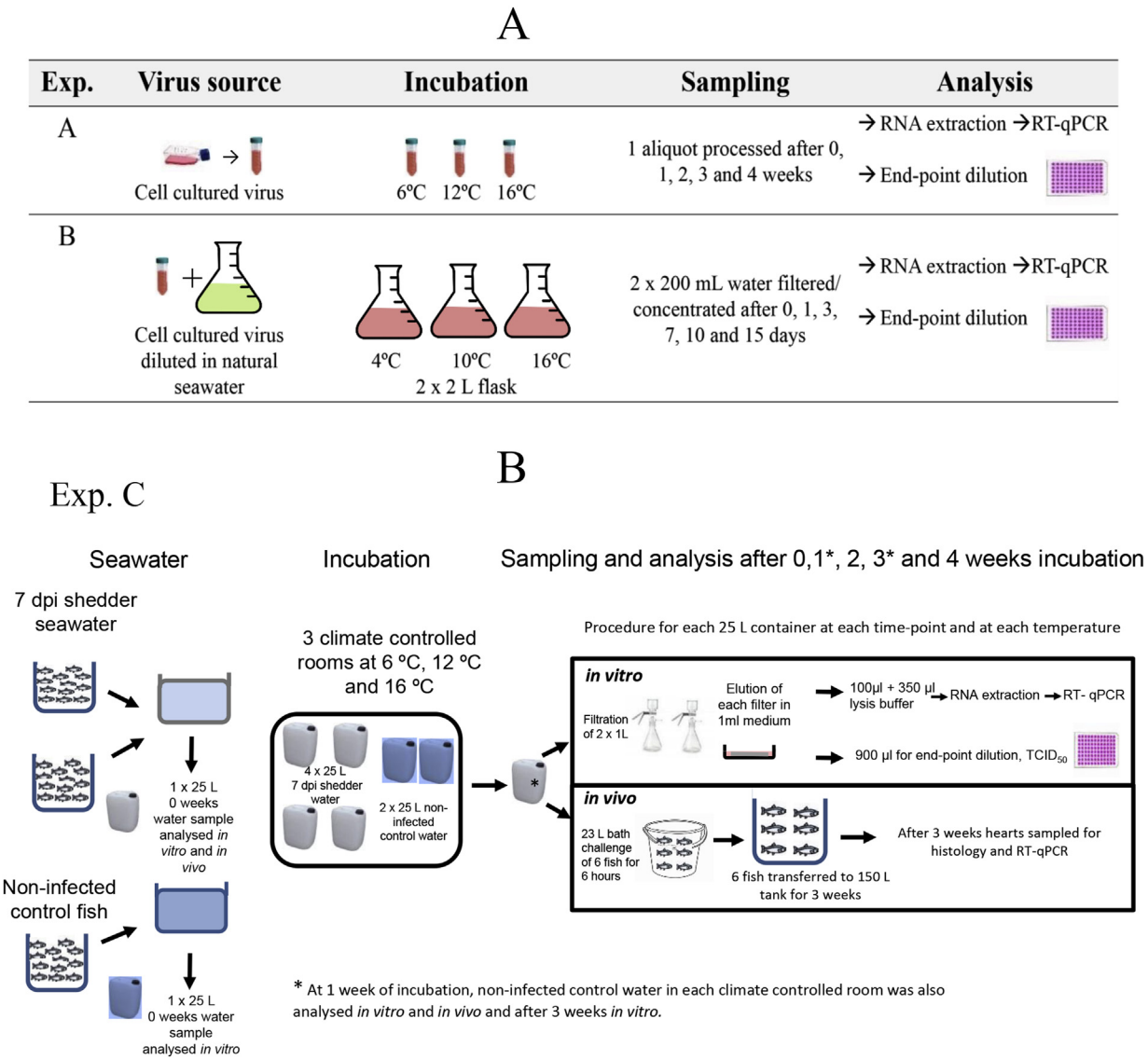

Fig. 1. Experimental design. A) Experiments A and B including, the source of SAV3, incubation temperatures, sampling points, and assays used. **B) Experiment C** including production of seawater containing SAV3 (SAV seawater), incubation set-up and sampling and analysis.

temperatures. It was analysed over 4 weeks for the survival of SAV using the *in vitro* methods mentioned above in addition to an *in vivo* method: immersion of naïve salmon in this seawater. Detailed information for each experiment has been described below.

2.3.1. Experiment A: Survival of cultured SAV incubated at different temperatures (in vitro, Fig. 1A)

In this experiment four aliquots of cultured virus stock in 2-mL cryotubes (polypropylene, Nunc) were incubated in each of the 3 climate-controlled rooms at 6, 12 and 16 °C, altogether 12 aliquots. An aliquot was removed from each incubation temperature at 1, 2, 3, and 4 weeks post-incubation. One hundred microlitres of each aliquot was then transferred into a microtube containing 350 μL of lysis buffer (iPrep™ PureLink™ Total RNA Kit, Invitrogen, USA) and stored at −80 °C prior to RNA extraction and RT-qPCR analysis. The remaining 1.8 mL was analysed to check infectivity on CHH-1 cells using 100 μL of each dilution from the dilution series per well and the plates were incubated at 15 °C for 7 days. Cultured SAV3 stored at 4 °C was used as a positive control (a temperature where no deterioration of virus was expected) and L-15 medium was used as a negative control on these TCID50 plates. TCID**50** titre was calculated after 7 days using the Spearman and Karber method (Ramakrishnan, 2016). Experiment A was carried out simultaneously with Experiment C.

2.3.2. Experiment B: Survival of cultured SAV in seawater incubated at different temperatures (in vitro, Fig. 1A)

To determine SAV survival in seawater at different temperatures, cultured SAV was mixed in 6x2L of seawater in conical glass flasks. Bacterial and fungal growth was necessarily controlled by the addition of Penicillin-Streptomycin-Amphotericin B (PSA) at concentrations recommended for cell culture. The flasks in duplicate were incubated at 4, 10, and 16 °C in 3 separate incubators. In this experiment, seawater with PSA, but no virus incubated at 16 °C was used as a negative control. Seawater samples (200 mL) from each of the duplicate glass flasks were collected after 0, 1, 2, 3, 7, 10, and 15 days of incubation and filtered as described in the section for viral analytical methods. Both eluant from filtered seawater at 16 °C and L-15 medium were used as negative controls and analysed using the TCID50 assay. The virus stored at 4 °C was again used as a positive control on the TCID50 plates. Supernatants from the TCID50 plates from all treatments at two selected sampling points (day 10 and 15) were randomly selected and checked for detection of SAV3 RNA using RT-qPCR to verify that any observed cytopathic effect was due to SAV rather than cytotoxicity.

2.3.3. Experiment C: Survival of SAV shed by SAV-infected fish incubated at different temperatures (in vitro and in vivo, Fig. 1B)

One hundred and thirty Atlantic salmon post-smolts (after 5 days in seawater) were injected (*i.m.*) with 0.1 mL containing 10^3 TCID50 of SAV cultured in CHH-1 cells. These shedder fish were maintained in two 250-L tanks (65 fish per tank) and fish husbandry was as described above. On day 7 post injection (experimental time 0), the seawater from these 2 tanks was pooled to provide approximately 300-L of seawater containing SAV. This pooled seawater was then aliquoted into twelve 25-L containers (food quality HDPE, Polimoon Packaging Ltd. UK) and 4 containers were incubated at each chosen temperature (6, 12, and 16 °C) in climate-controlled rooms. For control seawater, 20 untreated fish were reared in approximately 200 L seawater in a 250 L tank for 7 days. Approximately 150-L seawater from this tank was dispensed into six similar 25 L containers and 2 containers of this control seawater were incubated alongside the SAV seawater at each temperature. All containers with water were periodically (every 2–3 days) aerated using compressed air to prevent stagnation and to help mimic the dynamic marine environment. At the experimental time 0, three randomly selected shedder fish from each tank were sampled for RT-qPCR (heart) and histology (heart, and pancreas) to confirm that the shedder fish were infected and had contributed towards the shed virus.

This SAV seawater was analysed at time 0, which represents the starting titre. After 1, 2, 3 and 4 weeks of incubation, one 25 L-container with seawater containing SAV was removed from each of the three chosen temperatures from the climate-controlled rooms and was analysed using both *in vitro* (TCID50 and RT-qPCR) and *in vivo* (naïve Atlantic salmon post-smolts bath challenged) methods (Fig. 1B). Six salmon smolts were exposed to this incubated virus containing seawater per temperature per incubation period and maintained in seawater tanks for three weeks before being euthanized and sampled. As the virus containing seawater was taken out weekly, the exposure was carried out weekly, and each batch of exposed fish were maintained in their respective separate tanks, altogether 16 tanks started and terminated sequentially each tank with fish maintained for 3 weeks.

For the *in vitro* test, 2 x 1 L of seawater was filtered/concentrated as described in the viral analytical methods. The negative control seawater was only processed at 1 and 3 weeks of incubation. This filtered/concentrated control seawater was used on TCID50 plates in addition to L-15 medium as a negative control at the time-points when it was processed (1 and 3 weeks post incubation).

For the *in vivo* test, the remaining 23 L of virus containing seawater at all four weeks and control seawater at 1 week post incubation was used for the bath challenge of six recently seawater transferred fish (5 days after seawater transfer) in 80 L containers for 6 h. The seawater was aerated and oxygen levels carefully monitored during the bath challenge and after 6 h the 6 challenged fish bathed in water from each temperature were transferred into a 150 L tank. Each group of 6 fish were maintained for 3 weeks post challenge before being euthanized and sampled. Hearts were halved and ventricle with bulbus arteriosus was snap frozen for subsequent RNA extraction and RT-qPCR analysis. The other half of each heart including the atrium and the pyloric caeca including pancreas were fixed in 10% neutral buffered formalin for histology.

2.4. RNA extraction and RT-qPCR

Total RNA was extracted from heart tissue with TRIzol® reagent (Ambion) and PureLink™ Total RNA Kit (Invitrogen, USA) in an iPrep™ machine. RNA was extracted from filtered/concentrated seawater samples and supernatant samples from TCID50 plates using lysis buffer and PureLink™ Total RNA Kit according to the manufacturer's instructions. The extracted RNA in a final volume of 50 μL was quantified using a NanoDrop™-1000 spectrophotometer (Thermo Fisher Scientific).

Seawater samples were spiked with nodavirus as an internal control (Korsnes et al., 2005) to validate the recovery from the RNA extraction and the subsequent cDNA synthesis. All the samples tested showed a satisfactory level of nodavirus.

An RT-qPCR assay targeting the SAV nsP1 gene for detection of SAV with the following modification of the probe sequence FAM-5′-TCGA AGTGGTGGCCAG-MGB (Andersen et al., 2007) was performed in a one-step assay for heart samples and a two-step assay for water eluants and TCID50 supernatant samples, as described below. Two hundred nanograms of total RNA from heart tissues were analysed using AgPath-ID™ One-Step RT-qPCR Reagents (Thermo Fisher Scientific) in a total volume of 10 μL with 400 nM of forward primer and 600 nM of reverse primer, and 160 nM of the probe in 96 well-plates. cDNA synthesis was performed from filtered seawater eluant and cell culture supernatant samples, using 2 μL total RNA in a 10 μL reaction volume using SuperScript VILO cDNA Synthesis Kit (Thermo Fisher Scientific). Then, 2 μL of synthesized cDNA were used in a 10 μL reaction mix with TaqMan™ Fast Universal PCR Master Mix (2X), no AmpErase™ UNG (Thermo Fisher Scientific) and 900 nM each of forward and reverse primer, and 250 nM of the probe on 96 well-plate. RT-qPCR assays were performed using ABI 7900HT Fast Real-Time PCR system (Thermo Fisher Scientific) and the temperature profile was adjusted as follows; activation at 95 °C for 20 s followed by 40 cycles of denaturation at

95 °C for 10 s, and annealing and extension at 60 °C for 20 s. Copy numbers of the nsp-1 amplicon were calculated by the Applied Biosystems SDS software for all samples by using a 6 point standard curve of an RNA fragment (576 bases of the nsp-1 gene) generated using ThermoScript™ RT-PCR System and Platinum® Taq DNA polymerase (Invitrogen, USA.) and primers incorporating the T7 promotor sequence in the forward primer. Purified PCR products were *in vitro* transcribed using T7MEGAscript Kit (Ambion) at 37 °C for 16 h followed by DNase treatment (TURBO DNA-free™ Kit, Ambion) (Jarungsriapisit et al., 2016a). NTCs were included in each RT-qPCR run and a threshold value of 0.1 applied to all data.

2.5. Histology

Heart and pancreas tissues of fish challenged in Experiment C (Fig. 1B) that were positive for SAV using RT-qPCR and the few randomly selected shedders were processed for histology as described by Jarungsriapisit et al. (2016b). The tissue sections were examined under light microscope for PD histopathological lesions using a modified scoring system published earlier (Andersen et al., 2010; Jarungsriapisit et al., 2018).

3. Results

3.1. Experiment A: Survival of cultured SAV3 incubated at different temperatures (in vitro)

A decrease in SAV titres from 7.6×10^4 TCID50 mL^{-1} was observed over time at both 12 and 16 °C after 4 weeks of incubation. At 6 °C SAV titres were stable within 1 log, between 1.5×10^4–7.6×10^4 TCID50 mL^{-1} throughout the incubation period with a half log decrease at 4 weeks. At 12 °C, SAV showed a decrease in titre from 7.6×10^4 to 3.4×10^3 TCID50 mL^{-1} after 3 weeks of incubation and the titre remained at 4.0×10^3 TCID50 mL^{-1} at 4 weeks of incubation. At 16 °C, SAV titres decreased more drastically than at 12 °C from 7.6×10^4 TCID50 mL^{-1} to 1.6×10^3 TCID50 mL^{-1} at 3 weeks of incubation with a final titre of only 79 TCID50 mL^{-1} at 4 weeks of incubation (Fig. 2A). The copy numbers of SAV3 RNA were between 1.6×10^5 and 3.3×10^4 with no specific trend at any of the tested temperatures (Fig. 3A, Supplementary 1A). All control samples were negative for SAV analysed both by TCID50 assay and by RT-qPCR.

3.2. Experiment B: Survival of cultured SAV in seawater and incubated at different temperatures (in vitro)

Survival of cultured SAV in seawater incubated at 4, 10, and 16 °C was investigated *in vitro,* using CHH-1 cells. The titres of SAV decreased over time at all temperatures (Fig. 2B). At 4 °C, SAV titre was maintained at 3.3×10^4 TCID50 mL^{-1} to 3.0×10^4 TCID50 mL^{-1} at 7 days of incubation, which decreased to 1.6×10^3 and 2.6×10^3 TCID50 mL^{-1} at 10 and 15 days of incubation, respectively. At 10 °C, the decreasing trend of SAV titre was similar to the trend at 4 °C resulting in the lowest titre of 8.5×10^2 TCID50 mL^{-1} at 15 days of incubation. At 16 °C, SAV titre decreased to 4.0×10^3 TCID50 mL^{-1} at 7 days of incubation and was undetectable at 15 days of incubation. The copy numbers of SAV3 were between 1.9×10^3 and 3.0×10^3 at 4 °C and 10 °C and only the seawater containing SAV incubated at 16 °C showed signs of declining viral RNA at 15 days of incubation (Fig. 3B, Supplementary 1B).

3.3. Experiment C: Survival and infectivity of SAV shed by SAV-infected fish incubated at different temperatures (in vitro and in vivo)

Histology of shedder fish showed necrosis of pancreatic exocrine cells and myocarditis which are typical histopathological lesions of PD (Fig. 4A and B). This along with the positive detection of SAV RNA in hearts of the shedder fish verifies that the fish were infected. Virus was detected in seawater from tanks with the shedder fish at 7 dpi (882 TCID50 L^{-1}) which constitutes the peak of the viraemic period of the shedder fish (7 dpi) (Jarungsriapisit et al., 2016b), Water was collected

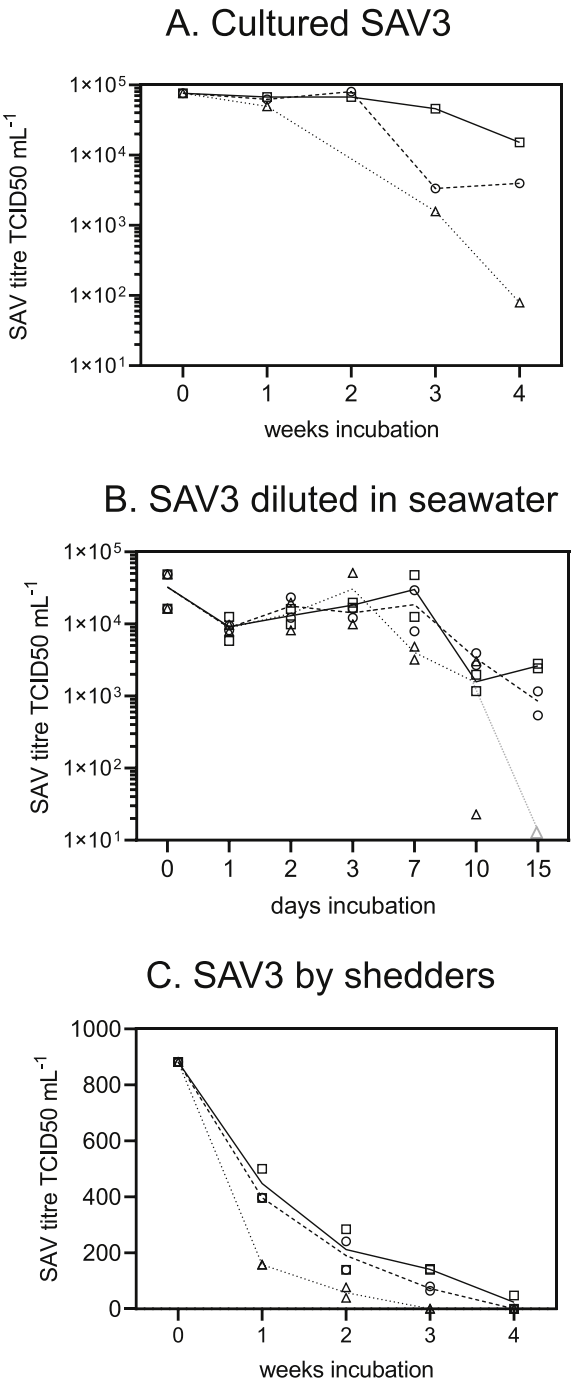

Fig. 2. Survival of SAV incubated at different temperatures detected by TCID50 assay (For Exp B and C, n = 2, both replicates are plotted with an average trend-line). A. Cultured SAV incubated at 6 °C (open square, □ and solid line), 12 °C (open circle, ○ and dashed line), and 16 °C (open triangle, Δ and dotted line), n = 1; **B.** Cultured SAV in seawater incubated at 4 °C (open square, □ and solid line), 10 °C (open circle, ○ and dashed line), and 16 °C (open triangle, Δ and dotted line), the last point at 16 °C (grey triangle, △ with stippled grey line) represents duplicate values below the detection limit; **C.** SAV shed by SAV-infected fish, incubated at 6 °C (open square, □ and solid line), 12 °C (open circle, ○ and dashed line), and 16 °C (open triangle, Δ and dotted line). Note that y-axis of A and B are in log scale, and there is no data of cultured SAV in 2A at 2 weeks of incubation at 16 °C due to a technical problem.

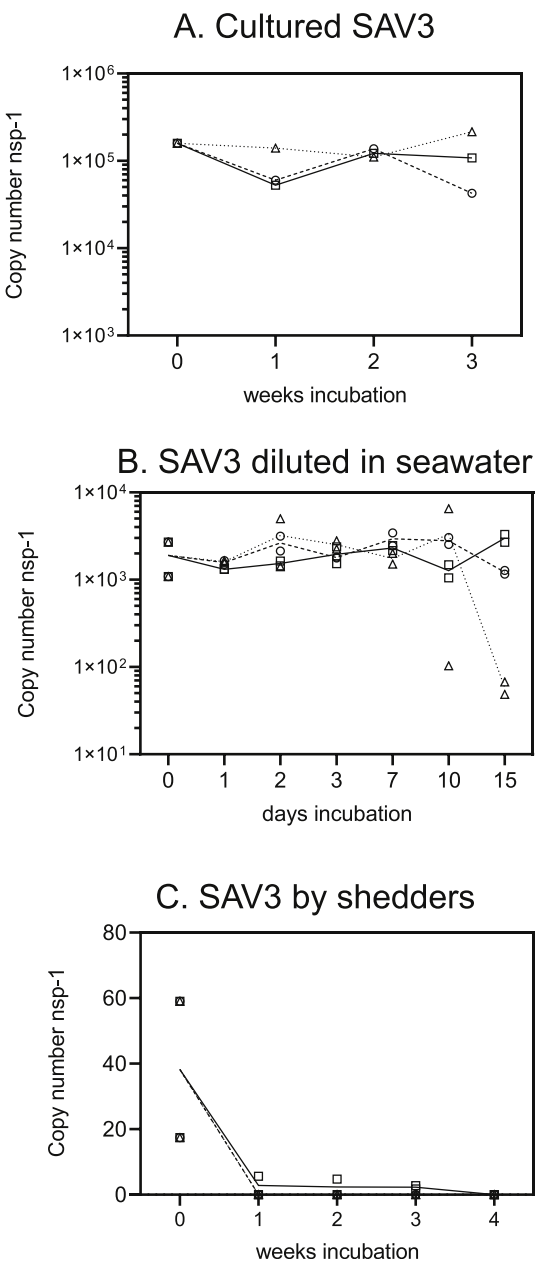

Fig. 3. Copy numbers of SAV RNA from SAV virus incubated at different temperatures (For Exp B and C, n = 2, both replicates are plotted with an average trend-line). A. Cultured SAV incubated at 6 °C (open square, □ and solid line), 12 °C (open circle, ○ and dashed line), and 16 °C (open triangle, Δ and dotted line). No data at 4 wpi due to technical reasons. **B.** Cultured SAV in seawater incubated at 4 °C (open square, □ and solid line), 10 °C (open circle, ○ and dashed line), and 16 °C (open triangle, Δ and dotted line), **C.** SAV3 shed by SAV-infected fish, incubated at 6 °C (open square, □ and solid line), 12 °C (open circle, ○ and dashed line), and 16 °C (open triangle, Δ and dotted line).

from the tanks at this time and represents the time 0 titre at the beginning of survival Experiment C, 882 TCID50 L^{-1} seawater.

After incubation at 6, 12, and 16 °C for 1 week, the SAV titres decreased to 448, 397, and 158 TCID50 L^{-1} seawater, respectively. At two weeks after incubation, SAV3 titres at 6, 12, and 16 °C were decreased to 212, 190, and 58 TCID50 L^{-1} seawater, respectively. Further decrease in titres were observed at 3 weeks of incubation at 6 °C, and 12 °C to 141, and 73 TCID50 L^{-1} seawater, respectively, whereas infectious SAV at 16 °C was undetectable. At 4 weeks of incubation, detection of SAV3 was only detectable at 6 °C with 24 TCID50 L^{-1} seawater (Fig. 2C). Copy numbers of SAV3 RNA at time 0 were 42, but thereafter could only be detected in seawater incubated at 6 °C for up to 3 weeks post incubation (Fig. 3C, Supplementary 1C).

At time 0, the day the shedder water was collected, 6 fish were bath challenged in this seawater containing SAV from shedder fish. These 6 fish were sampled 3 weeks post challenge and 5 of the 6 were positive for SAV RNA in their heart samples (Table 1). This prevalence dropped dramatically to 2 of 6 bath challenged fish for shedder water incubated for 1 week at 6 °C. Histological examination of these 2 positive fish showed few histopathological changes in heart while they had normal histology of pancreas (Fig. 4). At this time-point the fish bathed in control water from all temperatures were all negative for SAV RNA and control water was consequently only analysed *in vitro* at 3 weeks post incubation. At later time points, fish bath challenged with SAV water incubated at all temperatures were negative for SAV RNA (Table 1). Histopathological changes in the shedder fish were more severe compared to the challenged fish (Fig. 4).

4. Discussion

As aquaculture in open cages is the dominating production method, there is a high probability of pathogens being released into the environment during an outbreak. Information about pathogen transmission is crucial for risk assessment and the strict preventive measures implemented by regulatory authorities. The need for this type of information that supports better management is also driven by the increased public concern of the environmental impact of diseases in fish farming. The results from this study add to the knowledge necessary for risk assessment during PD outbreaks for both farmed salmon and wild salmon.

Cell cultured SAV3, cell cultured SAV3 mixed in seawater, and SAV3 in seawater shed by SAV3-infected fish incubated at different temperatures were examined for their viability and infectivity by employing a TCID50 assay on CHH-1 cells (*in vitro*). Since SAV3 shed by infected fish best represents virus shed by fish in sea cages in the field and might contribute to the disease transmission, the viability of SAV3 shed by infected fish and incubated at various temperatures was also used in a bath challenge of recently seawater-transferred post-smolts (*in vivo*). To the best of our knowledge, this is the first report investigating SAV viability and infectivity in seawater containing SAV shed by viraemic salmon using both CHH-1 cell lines (*in vitro*) and using recently seawater-transferred post-smolts (*in vivo*).

The TCID50 assay is a useful tool to study disease transmission that measures the number of virus particles capable of infecting and replicating in cells, although it is labour-intensive and time-consuming. In contrast, RT-qPCR is a rapid and efficient method, but it is unable to distinguish between infectious and non-infectious virus as it detects only the nucleic acid. Interestingly, the TCID50 assay proved more sensitive than RT-qPCR, especially at low concentrations of the virus, as previously reported (Jarungsriapisit et al., 2016a). This is probably due to a combination of a greater volume being applied per well, and the use of several replicate wells, thus more material altogether is being tested in the TCID50 assay. In addition, viral multiplication during the development of CPE on the cell culture plates could account for the increasing sensitivity of the TCID50 assay especially at low virus concentrations. Not surprisingly the copy number assay showed that the nucleic acid component of SAV3 is a lot more stable than the infectious qualities of SAV3 and illustrates the necessity of at the very least TCID50 assay for survival studies.

The starting titres of virus were different in the three experiments. The relatively high titre in Exp. A could have been diluted in cell culture medium to resemble that in Exp. B, but it was important to be able to measure the changes in virus concentration and this becomes increasingly difficult to do so accurately with diluted virus. Although, the SAV3 in the seawater (Exp. C) had the lowest starting titre as this was dependent of shedding of virus from shedder fish, and therefore became undetectable at the earliest sampling points the *in vivo* data it provided

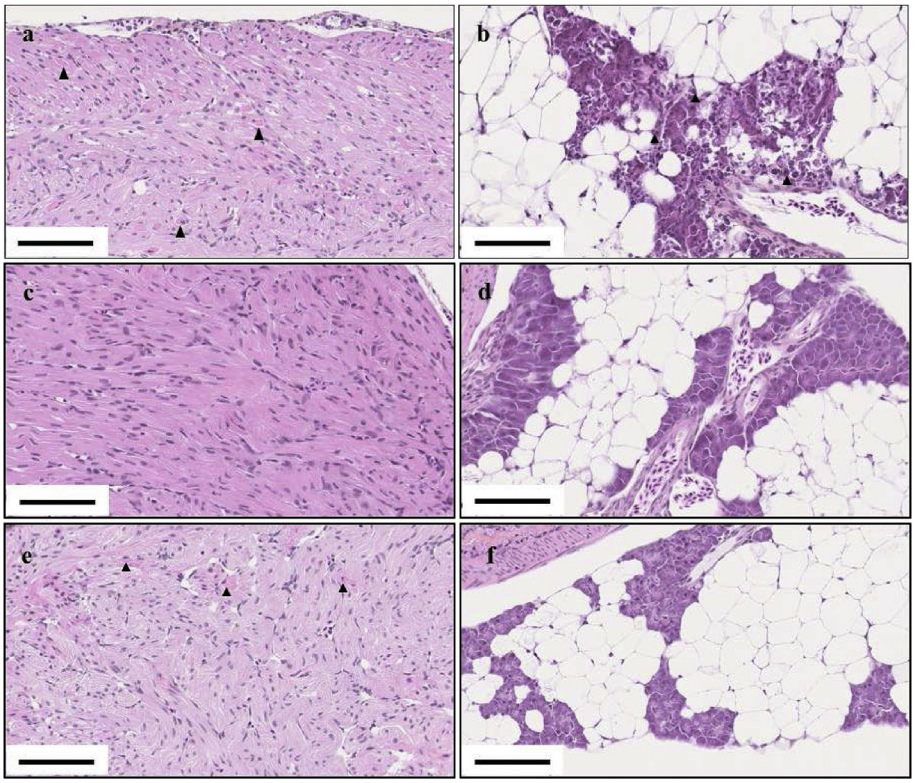

Fig. 4. Histopathological changes in the heart (a, c, and e) and pancreas (b, d, and f) of shedders (a and b) and fish that were positive after challenge with shed virus (c–f). a: heart from a shedder showing severe diffuse myocardial degeneration (arrowheads), **b:** pancreas from a shedder showing significant multifocal necrosis of exocrine cells (arrowheads), **c:** Normal appearance of heart from fish 1 exposed to seawater containing virus incubated at 6 °C for 1 week, **d:** Normal appearance of pancreas from fish 1 exposed to seawater containing virus incubated at 6 °C for 1 week, **e:** heart sample of fish 2 exposed to seawater containing virus incubated at 6 °C for 1 week. Focal myocardial degeneration ± inflammation (arrowheads), **f:** pancreas sample of fish 2 exposed to seawater containing virus incubated 6 °C for 1 week showing normal appearance. Bars represent 50 μm.

Table 1
Number of positive fish bath challenged with seawater containing SAV shed by shedder fish, incubated over time at different temperatures and analysed by RT-qPCR.

Treatment	Time 0	1 wpi	2 wpi	3 wpi	4 wpi
6 °C	5/6[a]	2/6[a]	0/6	0/6	0/6
12 °C		0/6	0/6	0/6	0/6
16 °C		0/6	0/6	0/6	0/6

[a] Positive fish had Ct values low enough to suggest clinically disease (22–24).
wpi = weeks post infection.

is the most relevant information for management of disease outbreaks in aquaculture. In Exp. B, the starting titre was higher and therefore its survival could be more accurately followed over time, but the need to add PSA to control growth of microorganisms due to nutrition coming from culture medium along with the cultured virus could have impacted the results. In contrast, in Exp. C seawater did not require PSA to inhibit the growth of microorganisms, thus any impact of PSA on the results could be avoided.

We could conclude that the viability of SAV3 is negatively affected by increasing temperatures whether the virus originates from cell culture or shed by viraemic fish. This conclusion is supported by earlier studies, on the viability of fish viruses including SAV, viral haemorrhagic septicaemia virus (VHSV), infectious pancreatic necrosis virus (IPNV), and nervous necrosis virus (NNV) exposed to different parameters. These studies have been performed *in vitro* using fish cell lines (Frerichs et al., 2000; Graham et al., 2007; Hawley et al., 2008; Tu et al., 1975; Villoing et al., 2000). Even though other parameters, such as organic matter, suspended solids, pH have not been included in the present study, these factors may also influence the viability of fish viruses (Garver et al., 2013; Graham et al., 2007; Oidtmann et al., 2017; Villoing et al., 2000). Initial tests for total carbon content of the seawater supply to the facility which has been used in these studies were low, which leads us to speculate that the SAV survival was not influenced by organic load. Previous studies have been performed using SAV subtypes 1 and 2 (Graham et al., 2007; Villoing et al., 2000). Since survival depends on such properties as membrane integrity and basic virus structure, it is likely that results for other SAV subtypes could be applied to SAV3 and *vice versa.* The differences between subtypes are not structural and good cross-neutralisation occurs between sub-types 1–5 (Graham et al., 2014).

The cultured SAV3 when incubated in the cell culture medium was relatively stable as expected, especially at lower temperatures. However, it does not accurately represent the virus shed from infected fish at the marine sites. Nevertheless, it may represent the survival of virus in dead fish or fish tissues. Infectivity of cultured SAV in seawater appears to decrease more abruptly compared to the virus shed by fish in seawater. It has been reported that the characteristics of viruses may change based on the cells they propagate in and this can influence their ability to infect (Pham, 2014). Therefore, there might be differences in viral properties of cultured virus compared to shed virus and these differences could explain this result. Since the biological properties of virus shed by infected fish more accurately represents virus released in connection with a disease outbreak, experimental designs using the shed virus should also be considered in any further studies of virus survival and disease transmission. Nevertheless, the *in vitro* assay is definitely useful for preliminary studies as it follows the principle of the 3Rs (Design of live animal experiments should reduce, refine and replace).

In the present study (Exp. C), the water that had been incubated at 6 °C for 1 week, had a concentration of 448 $TCID_{50}$ SAV3 L^{-1} of seawater, and successfully transmitted SAV in a bath challenge of recently seawater-transferred post-smolts. However, from 2 weeks, even though the TCID50 SAV3 L^{-1} of seawater remained higher than our previously published minimum infectious dose of 7 $TCID_{50}$ SAV3 L^{-1} (Jarungsriapisit et al., 2016a), none of the challenged fish tested positive for SAV. External environmental factors, in this case, temperature and time of incubation had clearly affected the virus's ability to infect fish compared to the freshly shed virus at time 0. Virus may appear significantly infectious using a TCID50 assay but may fail to infect susceptible fish indicating that the virus could have undergone changes

that make it more difficult to infect a host than an optimised cell culture. It is also possible that either the virus was taken up by the challenged fish, but it was then cleared by the fish immune system or the virus was not taken up during the exposure. In addition, although the ratio of fish to water volume was similar to previous studies (Jarungsriapisit et al., 2016a; Moore et al., 2017) with only 6 fish employed in the bath challenge compared to 55–61 fish, the sensitivity of this *in vivo* assay was not optimal. This may also explain why more infected fish were not found in the *in vivo* assay.

In our previous study (Jarungsriapisit et al., 2016a), the maximum shedding rate of post-smolts after a bath challenge with a high dose of SAV3 at 10 days post infection was estimated to be 2.4×10^4 $TCID_{50}$ h^{-1} kg^{-1}. Interestingly, the shedding rate of shedder fish after *i.m.* injection at 7 dpi in this study is estimated to be 2.3×10^4 $TCID_{50}$ h^{-1} kg^{-1}. These remarkably consistent values indicate that infected fish populations with a similar prevalence of viraemic fish (nearly 100%) and similar parameters, such as genetics, weight, age and conditions (i.e. recently seawater transferred fish), shed a similar amount of virus. Shedding also continues over a number of days (Jarungsriapisit et al., 2016b). Applying this example of shedding rate to a few hundred thousand of fish in a sea cage could mean, up to 10^8 $TCID_{50}$ SAV3 h^{-1}could be released into a sea cage if recently seawater-transferred post-smolt are infected with SAV. At the same time, a lower prevalence of viraemic fish in the sea cage, large volumes of seawater, and seawater currents could dramatically lower the concentration of the shed virus in the field. However, both this study and previous studies have shown that SAV3 is extremely infectious and can cause infection at low concentrations. Earlier studies have examined the impact of UV light and organic loads on survival of virus (Stene et al., 2016; Vike et al., 2014). Other parameters such as prevalence of viraemic fish, the biomass of the fish at the site, shedding rate, volume of seawater, wind speed, and seawater currents, are important for increasing the accuracy of mathematical models that may be used as important tool for risk assessment (Salama et al., 2011; Viljugrein et al., 2009; Stene et al., 2014). Although, there would be uncertainties in the estimation due to factors such as differences in disease progression in fish individuals, virus concentration and virus dilution, these results concerning the survival of SAV3 in natural seawater at different temperatures could support a more realistic hydrodynamic model. Modelling for risk assessment during PD outbreaks is dependent on accurate information about several parameters to produce a realistic risk. Such modelling would be helpful in avoiding risks to wild fish and to fish in aquaculture in the near vicinity and further afield as they may be exposed to shed virus from locations with a PD outbreak, contract infections and given the right conditions develop disease. The results indicate that SAV retains infectivity at low temperatures for at least a week and could be transported over long distances during this time.

The present study adds knowledge to the field of SAV3 transmission. The amount of SAV3 necessary for disease transmission to Atlantic salmon post-smolts when exposed to different temperatures, and the time required for virus inactivation have been estimated. We have shown that shed virus after being in the environment is less infective to fish compared to the freshly shed virus. This suggests that terms such as non-infectious and total inactivation must be accompanied by the assay methods used for the studies. Also, since the TCID50 assay appears to be more sensitive than the *in vivo* method used in this study, it possibly overestimates the risk of transmission to wild fish in the environment and cultured fish at other locations. Therefore, data from TCID50 analysis could represent a worst-case scenario during risk assessment.

Future studies should optimally concentrate on using such parameters as the prevalence of viraemic fish and calculating the shedding rate. In order to support risk assessment in the field this information could also be incorporated into mathematical transmission models for epidemiological studies in order to understand the spread of SAV3 and enveloped viruses.

Conflicts of interest

The authors declare that they have no competing interests.

Authors' contributions

JJ, SP, LJM and JN participated in designing the experiment. JJ, SP, JN, and NNO carried out post-smolt challenge with seawater containing SAV3. JJ, SP, LJM and NNO collected water and fish samples. JJ and NNO performed virus concentration from seawater and quantification of SAV3 by TCID50 assay. JJ, NNO and SM conducted RT-qPCR assay and calculation of RNA copy number. JJ, SP, NNO, LJM and SM interpreted the results, and wrote the manuscript. All authors critically revised the manuscript, read, advised, took part in discussion, and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2019.734647.

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