

Survival of Salmonid alphavirus in seawater under different physical conditions

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Pål Skjold



Department of Biology
University of Bergen
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Abstract

Pancreas disease (PD) is an infectious disease caused by Salmonid alphavirus (SAV), which infects Atlantic salmon, *Salmo salar* L., and rainbow trout, *Oncorhynchus mykiss* (Walbaum). PD causes substantial economical losses in salmonid aquaculture in Europe, and has, since the early 2000s, steadily increased in extent in Norwegian aquaculture. Knowledge concerning transmission pathways for the causative agent is of paramount importance in order to combat the disease. Many authors have emphasized horizontal transmission as the most important pathway for infecting new cohorts and hydrodynamic models have been used to investigate if waterborne transmission by passive drift by ocean currents could explain why PD outbreaks has a tendency to cluster in time and space. SAV RNA has been detected in a lipid film, often seen in and around salmon farms, and this lipid film has therefore been proposed to contribute to dissemination of SAV between adjacent farms, driven by wind and currents. An important premise in such models is how long the virions remain infective in seawater outside its host. The survival of SAV in natural seawater and in seawater exposed to ultraviolet radiation (UVR) with wavelengths present in sunlight, simulating actual conditions in the natural environment, was investigated in this study.

SAV infectivity was examined in parallel beakers containing natural seawater, sterile seawater and sterile seawater with an oil layer over a time period of 72 hours. Equivalent parallels were additionally exposed to UVR. The UV index-value used in the experiment was measured to be 22 at the surface of the water and a mean value 11 in the beakers was calculated. At 10 sampling points, water samples were taken from each beaker and infectivity was tested in CHSE-214 cells. Water samples were also analysed by real-time RT-PCR to detect viral RNA.

In beakers containing sterile seawater, both with and without oil, infective virions were isolated throughout the experiment. In beakers containing natural seawater, the last infective virions were isolated at 48 and 24 hours after start, respectively. In beakers containing seawater and oil exposed to UVR, infective virions were isolated after 3 hours. In the other beakers exposed to UVR, infective virions were only isolated at the start of the experiment.

Based on the results in this study, it is anticipated that SAV virions will survive for less than 72 hours in the natural environment, given a seawater temperature around 10°C.

Abbreviations

CC: cell culture

CHSE: Chinook Salmon Embryo

CPE: Cytopathic effect

Ct-value: Cycle threshold-value

DAPI: 4',6-diamidino-2-phenylindole

dH₂O: Distilled water

EMEM: Eagle's Minimum Essential Medium

FBS: Fetal Bovine Serum

IFAT: Indirect (Immuno-) Fluorescence Antibody Test

IPNV: Infectious Pancreatic Necrosis Virus

ISAV: Infectious Salmon Anaemia Virus

mL: millilitre

μL: microlitre

MNE: Mean Normalized Expression

mRNA: Messenger Ribonucleic Acid

NE: Normalized Expression

nM: nano Molar

nm: nano meter

NSAV: Norwegian Salmonid Alphavirus

PBS: Phosphate Buffer Saline

Pers. comm.: Personal communication

RNA: Ribonucleic Acid

RT-PCR: Reverse Transcription-Polymerase Chain Reaction

SAV: Salmonid Alphavirus

TCID₅₀: Tissue culture 50 % infective dose

UV-A: 320-400 nm

UV-B: 280-320 nm

UV-C: 200-280 nm

UVR: Ultra violet radiation

Introduction

Pancreas disease (PD)

Pancreas disease is an infectious disease caused by Salmonid alphavirus (SAV), which infects Atlantic salmon, *Salmo salar* L., and rainbow trout, *Oncorhynchus mykiss* (Walbaum). The disease was first recognised on farmed Atlantic salmon in Scotland in 1976 (Munro et al., 1984), and then later in Norway and Ireland (Poppe et al., 1989, Murphy et al., 1992). The term pancreas disease was used because of the characteristic degeneration of the exocrine pancreatic tissue (Munro et al., 1984, McVicar, 1987). Although an infectious aetiology was suspected, it was first in the mid 90's that a Toga-like virus was isolated from salmon with clinical PD (Nelson et al., 1995). The virus was first named Salmon Pancreas Disease Virus (SPDV), but in 1999 SPDV was shown to belong to the genus *Alphavirus*, and the name Salmonid alphavirus was therefore proposed (Weston et al., 1999, Weston et al., 2002, Weston et al., 2005).

Today, six genogroups of SAV (1-6) have been identified (Fringuelli et al., 2008), and until 2010 only SAV 3 had been isolated from salmon in Norway (Hodneland et al., 2005, Karlsen et al., 2006, Jansen et al., 2010a). SAV 2, which had mainly been described from freshwater-reared rainbow trout in several countries in Europe (McLoughlin and Graham, 2007, Fringuelli et al., 2008), was in 2011 isolated from salmon in Norway from a number of outbreaks in seawater north of the enzootic zone for PD (Hjortaas et al., 2013). PD-outbreaks causes considerably economical losses, and has a major impact on Norwegian aquaculture (Aunsmo et al., 2010).

SAV characteristics

SAV is a member of the family *Togaviridae* and genus *Alphavirus* (Weston et al., 1999). The viral particles are described as enveloped and approximately 55-65 nm in diameter (Boucher et al., 1995, Nelson et al., 1995, Villoing et al., 2000). In general, Alphaviruses are small (45 to 75 nm in diameter), enveloped viruses with an icosahedral nucleocapsid core surrounded by a membrane bilayer (Strauss and Strauss, 1994, Garoff et al., 2004). The nucleocapsid consist of 240 copies of the capsid protein arranged in a icosahedral structure containing one copy of the single-stranded positive sense RNA genome (Strauss and Strauss, 1994, Garoff et al., 2004).

80 spikes comprising glycoproteins E1 and E2 are anchored in the membrane. E1 and E2 forms a stable heterodimer, and three heterodimers constitute one spike (Strauss and Strauss, 1994).

The SAV genome consists of approximately 11 900 nucleotides with four well-characterized conserved regions, as known for other alphaviruses (Weston et al., 2002). The full-length genome has a 5'-cap, a 3'-polyadenylated tail, and two open reading frames (ORF) (Knipe et al., 2007). The first ORF is approximately 2/3 of the genome length, and codes for the non-structural polyprotein P1234. The other ORF codes for the structural proteins C (capsid protein), E1, E2, E3 and 6K/TF (Firth et al., 2008). Both in the 5'- and 3'-end there is an untranslated region (UTR), in addition to one UTR between the two ORF's (Strauss and Strauss, 1994).

Alphavirus replication

It is likely that SAV uses the same pathway for entry, replication and budding as other more known alphaviruses (Karlsen et al., 2010). Alphaviruses has a broad range of hosts and have been found in many different tissues. It has therefore been suggested that the E2 glycoprotein either uses a ubiquitous receptor that is highly conserved across different species for entry, or that E2 has multiple binding sites that can bind to a wide range of receptors (Knipe et al., 2007, Jose et al., 2009). After binding, the virion gets internalized by receptor-mediated endocytosis (RME) (Strauss and Strauss, 1994). Inside the endosome, pH drops and E1 and E2 dissociates, exposing a fusion peptide on E1 that mix with the endosomal membrane and creates a fusion pore, allowing the nucleocapsid to enter the cytoplasm (Knipe et al., 2007, Jose et al., 2009, Kielian et al., 2010).

Once in the cytoplasm the nucleocapsid dissociates and releases the single-stranded genome, which both serves directly as messenger RNA for translation and as template for synthesis of the complementary negative strand (Hodneland, 2006). Ribosomes immediately associate with mRNA and translation of the non-structural polyprotein P1234 starts. The polyprotein subsequently gets cleaved to the negative strand replicase complex P123+nsP4 by the virus-encoded proteinase located within nsP2 (Knipe et al., 2007, Jose et al., 2009). Many Alphaviruses has an opal stop codon in the first ORF between nsP3 and nsP4, producing two polyproteins, P123 and P1234 in a ~6:1 ratio (Strauss and Strauss, 1994). No opal stop codon has been

identified in SAV (Weston et al., 2002, Hodneland et al., 2005). As P123 accumulates in the cytoplasm, the polyprotein becomes unstable and will be cleaved in nsP1, nsP2 and nsP3 because of the increased concentration of nsP2 proteases. nsP1-4 forms a new replicase complex that synthesizes positive sense subgenomic mRNA and new genomic RNA, using the negative strand as template (Strauss and Strauss, 1994). The subgenomic mRNA serves as template for translation of the structural polyprotein: capsid-pE2-6K-E1 (Hodneland, 2006). Immediately after the capsid protein has been translated, it is released by a serinelike protease that is located at the C-terminal of the protein (Knipe et al., 2007). The N-terminal of the new polyprotein now contains a signal sequence for translocation over the membrane of the endoplasmic reticulum (ER) for further processing (Strauss and Strauss, 1994). Newly synthesized capsid proteins quickly associate with genomic RNA to form new core particles (Strauss and Strauss, 1994). Inside ER, the polyprotein is proteolytically cleaved into pE2, 6K and E1. The pE2 protein is an intracellular precursor for the glycoprotein E2 and 6K is a transmembrane peptide carrying a signal sequence for E1 (Liljeström and Garoff, 1991). The pE2 and E1 proteins form a heterodimer that undergoes post-translational modifications, and are subsequently transported via the Golgi complex to the plasma membrane (Hodneland, 2006, Jose et al., 2009). Inside the Golgi complex, pE2 is cleaved by furin into E2 and E3 (Knipe et al., 2007, Jose et al., 2009). Recently a new structural protein was discovered, termed TransFrame (TF). A ribosomal -1 frameshift at a motif within the 6K sequence has been shown to occur, resulting in production of the TF protein (Firth et al., 2008). The function of the 6K and the TF proteins are not completely known, but they appear to play a role in envelope protein processing, membrane permeabilization, assembly and budding (Firth et al., 2008). Budding of new virions occurs when nucleocapsids assembled in the cell interact with membrane-bound E2 in a 1:1 ratio (Hodneland, 2006). As the nucleocapsid binds to the glycoproteins, the membrane is tightly pulled around the NC until it buds off (Knipe et al., 2007).

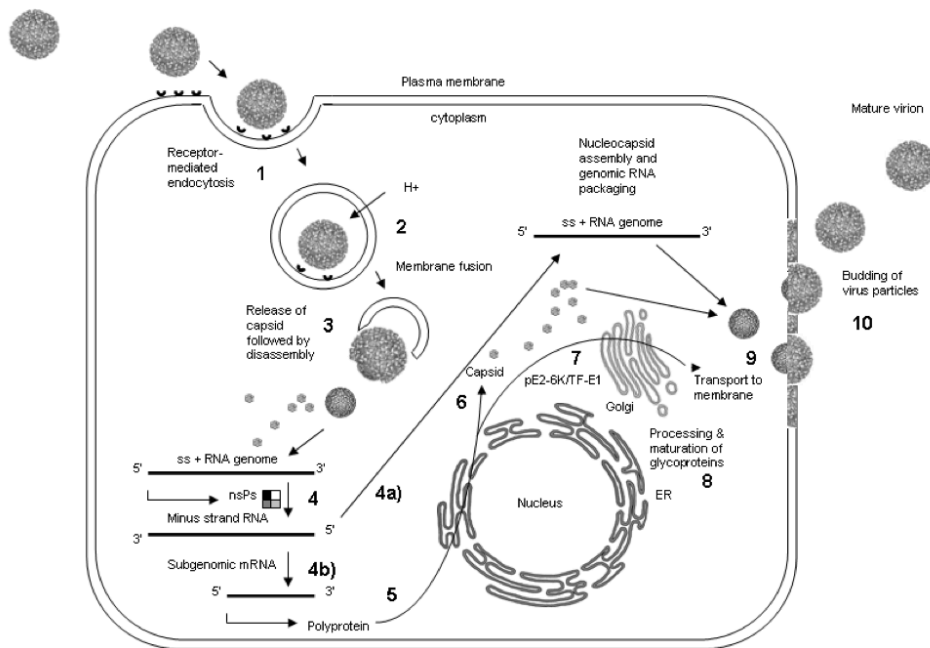


Figure 1. A schematic view of alphavirus entry, replication and budding in a host cell. Figure from (Andersen, 2012).

Clinical signs and pathology

During an outbreak of PD, the typical clinical signs appear as a drastic loss of appetite and fish become lethargic (McVicar, 1987). Darkening of the skin and increased number of faecal casts in the cages may also be observed (McLoughlin et al., 2002). There are no consistent gross pathological lesions seen during necropsy, but petechial haemorrhages in the pyloric area, pale heart, yellowish liver and ascites may be observed (McLoughlin et al., 2002, McLoughlin and Graham, 2007, Taksdal et al., 2007, Andersen, 2012).

For most SAV subtypes, the first histopathological changes can be seen as necrotic, or total loss of, pancreatic tissue (Munro et al., 1984, McLoughlin et al., 2002, Taksdal et al., 2007). Later in the pathogenesis, histological lesions can be found in heart and somatic muscle as degenerative myopathy, in addition to oesophageal muscle lesions (Ferguson et al., 1986). Fish that survive infection, but fail to regenerate pancreatic tissue, will become runts (McVicar, 1987).

Transmission

SAV is one of two aquatic Alphaviruses and the only known Alphavirus that infects fish, with the terrestrial Alphaviruses infecting mammals and birds (Andersen, 2012). Salmon louse (*Lepeophtheirus salmonis*) has been proposed as a potential vector for transmission of SAV, as for other known fish pathogens (e.g. ISA (Nylund et al., 1993)), as SAV RNA has been detected by real-time RT-PCR in salmon louse obtained from PD diseased salmon (Karlsen et al., 2006, Petterson et al., 2009). However, SAV has been shown to transmit between salmon without the presence of a vector (Boucher et al., 1995, McLoughlin et al., 1996), in addition, there is a negative correlation between occurrence of PD and the general number of salmon louse in Norwegian aquaculture (Petterson et al., 2009). Although all other known alphaviruses are arbovorne, i.e. they are transmitted through an arthropod vector (McLoughlin and Graham, 2007), salmon louse's contribution to transmission of SAV seems to be negligible in the bigger picture (Petterson et al., 2009).

Several studies have reported findings of SAV 3 in freshwater (Nylund et al., 2003, Karlsen et al., 2006, Bratland and Nylund, 2009), and under experimental conditions, all SAV subtypes have been shown capable of transmitting in freshwater (Graham et al., 2011). It is therefore possible that SAV 3 has a freshwater reservoir and thus, contributes to dissemination of the virus. Vertical transmission can also be a potential explanation, and has been suggested by some authors, but sufficient evidence for this has yet to be provided (Bratland and Nylund, 2009, Kongtorp et al., 2010, Jansen et al., 2010a). Nevertheless, today's knowledge about transmission of SAV emphasize the importance of horizontal transmission during the seawater phase (Fringuelli et al., 2008, Kristoffersen et al., 2009, Aldrin et al., 2010, Jansen et al., 2010a, Kongtorp et al., 2010, Snow et al., 2010), and hydrodynamic models have been found to correspond well with field observations on how SAV has spread from location to location (Viljugrein et al., 2009, Stene, 2013). These models do not, however, explain the whole picture, with e.g. sporadic outbreaks of PD in northern Norway far from the enzootic zone on the west coast.

Today, the farmed salmon itself is thought to be the main reservoir for SAV (Andersen, 2012). Jansen et al. (2010b) found that once SAV was detected by real-time RT-PCR at a site, all sampled individuals tested positive throughout the production cycle. And although the virus could not be cultured in cell culture at the later sampling points, these data could still indicate a possible persistent infection and

thus, a potential viral reservoir for the rest of the production cycle (Jansen et al., 2010b). However, SAV has also been detected in several types of flatfish in Scotland and Ireland (Snow et al., 2010, Bruno et al., 2014, McCleary et al., 2014), and phylogenetic studies of the six SAV subtypes suggest that the subtypes diverged long before farming of salmonids started, and that they therefore have been introduced separately from a wild reservoir, likely to exist in the North sea (Karlsen et al., 2014). Genetic distinct strains of SAV 3 have been isolated from salmon farms in close proximity, and genetic identical strains have been isolated from salmon farms separated by large geographical distances, indicating that waterborne, horizontal transmission can not alone explain the phenomenon that PD outbreaks have a tendency to cluster in time and space (Karlsen et al., 2014).

Ultraviolet radiation

Many authors have concluded that UVR is the most prominent factor contributing to viral decay in marine surface layers (Suttle and Chen, 1992, Cottrell and Suttle, 1995, Wommack et al., 1996, Noble and Fuhrman, 1997, Wilhelm et al., 1998). The nucleic bases in DNA and RNA within the virus are the main chromophores, i.e. the absorbing centres of UVR (Diffey, 1991, Jeffrey et al., 2000). UVR destroys the viruses by chemically modifying the nucleotides (Lytle and Sagripanti, 2005). While UVR can destroy the entire virion, a lethal damage to the nucleic acid will prevent the virus from infecting its host by making it unable to replicate (Suttle et al., 1993, Wilhelm et al., 1998). Other components in viruses can also absorb UVR, like proteins, but the absorbance is lower than that of nucleic acids (Diffey, 1991). RNA-viruses, as SAV, are in general more resistant to UVR because they lack the nucleic base thymine (Lytle and Sagripanti, 2005). However, strandedness also affects UVR sensitivity as double-stranded viruses exhibit higher resistance to UVR than single-stranded viruses (Rauth, 1965, Liltved et al., 2006). Infectious pancreatic necrosis virus (IPNV) is among the most UVR resistant viruses known, probably due to its double-stranded RNA (Liltved et al., 2006). Moreover, IPNV's RNA consists of relatively few base pairs (5 881 bp) (Dobos, 1995). This is of significance as the genome length is inversely proportional with UV-resistance, due to the amount of target molecules (Lytle and Sagripanti, 2005).

Table 1. Measured and predicted UV₂₅₄ (UV-C) sensitivities for three known fish viruses and their families. D₃₇ is defined as fluence producing on average one lethal hit per virion and reducing viable virus to 37% (Setlow et al., 1962). D is measured as fluence in J/m². Table modified from (Lytle and Sagripanti, 2005).

Family	Virus	Strand type	Genome size (kb)	Measured D ₃₇ (J/m ²)	Predicted D ₃₇ range for entire family (J/m ²)	References
<i>Birnaviridae</i>	IPNV	dsRNA	5.9 ²	120 (110-170)	110-120	(Liltved et al., 1995, Øye and Rimstad, 2001)
<i>Orthomyxoviridae</i>	ISAV	ssRNA	14.3 ³	7.5 (4.8-10)	7.3-11	(Øye and Rimstad, 2001)
<i>Togaviridae</i>	SAV ¹	ssRNA	11.9 ⁴	19 (7.3-23)	18-24	(Zavadova et al., 1968, Weiss and Horzinek, 1986, Smirnov et al., 1992)

¹UV₂₅₄ sensitivity for SAV has not been measured, but other viruses in the *Togaviridae* family (SINV, VEEV, SFV) have been tested and the value in the predicted D₃₇ range for the entire family is applicable for SAV. ²From Dobos (1995). ³From Kibenge et al. (2004). ⁴From Weston et al. (2002).

As shown in Table 1., IPNV has a remarkably higher resistance to UVR than both ISAV (Infectious salmon anaemia virus) and SAV. Liltved et al. (2006) reported similar findings when measuring differences in UVR resistance between ISAV and IPNV, where 75 J/m² was required for a 99.99 % reduction in viral titre for ISAV, compared to 2460 J/m² for IPNV. SAVs resistance to UVR has never been studied before, but as can be seen in Table 1., predicted D₃₇ for viruses in the *Togaviridae*-family are 18-24 J/m², thus, higher than ISAV. D₃₇ is defined as fluence (J/m²) producing on average one lethal hit per virion and reducing viable virus to 37% (Setlow et al., 1962). UV sensitivities in Table 1. are measured with wavelength 254 (UV-C) and are not directly applicable for nucleic acid damage induced by sunlight as irradiances under 290 nm are absorbed in the ozone layer, and thus do not reach the earth's surface (Diffey, 1991).

UV index

UV index is a unit of measure of UV irradiances in relation to effects on human skin (Vanicek et al., 2000). The UV index follows a linear curve, which means that a given value will cause twice the damage, with a given time, than half of the value, e.g. UV index 10 will cause the same damage during one hour as UV index 5 will cause in two

hours (B Hamre 2014, pers. comm., 4 Aug.). The UV index varies during the day, year and geographical position, and in countries near equator, UV index-values can reach up to 20 on a sunny day during the summer (Diffey, 2004). The viral inactivation by a given dose of UV radiation increases rapidly by decreasing wavelengths in the UV spectrum (Rontó et al., 1992). However, as irradiances from sunlight declines over the same wavelengths, a peak inactivation of viruses takes place around 300 nm in natural sunlight (Caldwell, 1971, Setlow, 1974, Figure 2).

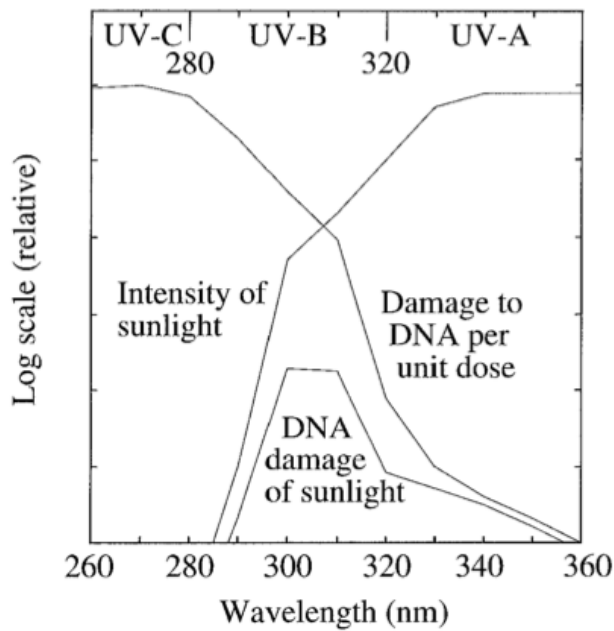


Figure 2. Interaction between intensity of sunlight and damage to DNA per unit dose. DNA damage of sunlight is estimated according to irradiances at sea-level (after Setlow, 1974). Figure from Murray and Jackson (1993).

SAV survival

It has been shown that SAV can remain infectious under experimental conditions for a long period in sterile seawater (Graham et al., 2007). However, bacterial biomass has been shown to be largely responsible for viral mortality in seawater, and the time outside a host is critical for viral survival (Murray and Jackson, 1992), which is consistent with markedly reduced SAV survival times in non-sterile seawater reported by Graham et al. (2007). A recent study reported findings of SAV RNA in oil leaking from dead PD diseased salmon, and suggested that this oil could contribute as protection and as a fomite during transmission of SAV from one salmon farming site to others (Stene et al., 2013). The speed of ocean currents are generally higher at the surface, often driven by wind friction (e.g. Garrison, 2007), and an oil layer

containing infective virions on the surface could therefore have a potential of spreading over longer distances than free virions in the seawater (Stene et al., 2013). Knowledge on how long SAV can remain infective in the natural environment is lacking, and there is no published work on SAV survival in natural seawater exposed to UVR, i.e. simulation of natural conditions. When considering the potential of waterborne, horizontal transmission of SAV in aquaculture, it is of paramount importance to know how long the virus can remain infective outside its host in the natural environment.

Aims of the study

The main objective of this study was to gain knowledge on the impact of sunlight (UV-B and UV-A) and microbiological activity on infectivity of SAV. Another aim was to identify the potential protection a lipid film, often seen in and around salmon farms, could provide the SAV virions against UVR.

Materials and methods

Cells and virus

Chinook salmon embryo (CHSE-214) cells were cultured in 175 cm² Nunclon™ flasks with 30 mL growth medium. The growth medium consisted of 500 mL Eagle's minimum essential medium (BioWhittaker®) supplemented with 50 mL Fetal Bovine Serum (10 %) (Sigma®), 5 mL Hepes Buffer (1 M) (Sigma®), 5 mL non-essential amino acids (100x) (BioWhittaker®), 5 mL L-glutamine (200 mM) (Sigma®) and 200 µL gentamicin sulphate (50.0 mg/mL) (BioWhittaker®). The cells were grown at 20 °C.

The SAV 3 isolate YK230812-7 was obtained from salmon during a PD outbreak on a salmon farm in Finnmark County in northern Norway in August 2012. Sterile filtered supernatant from third passage containing this isolate was used as inoculum.

Before the inoculum was added to the cell culture, the growth medium was discarded and 27 mL maintenance medium was added. The maintenance medium was identical to the growth medium except that it contained 10 mL (2 %) Fetal Bovine Serum (FBS) instead of 50 mL (10 %) as in the growth medium. The cell culture was washed twice with 1x phosphate-buffered saline (PBS) pH 7.3 before the maintenance medium was added. 3 mL of inoculum was then added to make a total volume of 30 mL in the flask. The cell culture was incubated at 14.5 °C for 2-3 weeks. Because cytopathic effect (CPE) is not consistently seen with SAV infection during the first passages in a cell culture (Jewhurst et al., 2004), this was not used as an indicator for infection, although the cultures were monitored regularly.

Pilot study

A pilot study was conducted prior to the survival experiment, mainly to make sure that the methods were working, and to get some practical training using the methods. Approximately 4 mL of sterile filtered supernatant with the SAV 3 isolate YK230812-7 was added to one litre of untreated seawater and stored in the dark at 4 °C. 50 mL SAV-containing seawater was sampled at four time points; after 75 min, 195 min, 24 hours and 48 hours. The water sample was filtered through an electropositive Zeta Plus® Virosorb® 1 MDS filter (Cuno Inc., U.S.A.) to concentrate

viral particles from the sample according to a VIRADEL (virus-absorption-elution) method as described by Andersen et al. (2010). Filtered virions were inoculated in cell culture flasks and incubated at 14.5 °C. After two weeks, RNA was extracted from supernatant from the cell cultures at all time points. RNA was also extracted from cells and supernatant from the cell culture used as inoculum. Real-time RT-PCR was performed on all samples.

The survival experiment

The survival experiment was carried out at the Department of Physics and Technology at the University of Bergen.

All supernatant from CHSE-214 cell culture flasks (infected with YK230812-7) was sterile filtered through filters of 1.2 µm, 0.45 µm and 0.2 µm respectively, one day prior to the start of the experiment. The supernatant was then gathered in a container before being allocated in 12 columns with approximately 22.5 mL supernatant in each. The columns were held at -80 °C over night. A subsample of 2 mL was stored at -80 °C in order to perform virus end point-titration.

The survival experiment was set up with 12 beakers, each containing 4 litres of water. 4 beakers contained untreated, natural seawater collected in the vicinity of several salmon farms in Bjørnefjorden in Os, Hordaland. 8 beakers contained seawater collected from the seawater supply at *Høyteknologisenteret i Bergen*. The in-house seawater was sterilized by autoclaving at 121 °C for 20 minutes. Both the natural and the sterile seawater used in the experiment was stored at 4 °C over night before the experiment was started. Four of the beakers with sterile water was added 115 mL of oil, which formed a layer of approximately 5 mm on the top of the water in those 4 beakers. The 115 mL of oil consisted of 92 mL (80 %) salmon oil (Ifex ehf.), 16 mL (14 %) rapeseed oil (AOR N.V., OiliO[®]) and 7 mL (6 %) fish oil (Amundsen[®]) from marine species. All beakers were given a Maxima 2 x 280 L/T aquarium O₂-pump to provide a minimum of circulation in the beakers and ensure that the inoculum was distributed evenly. The setup can be seen in Figure 3.

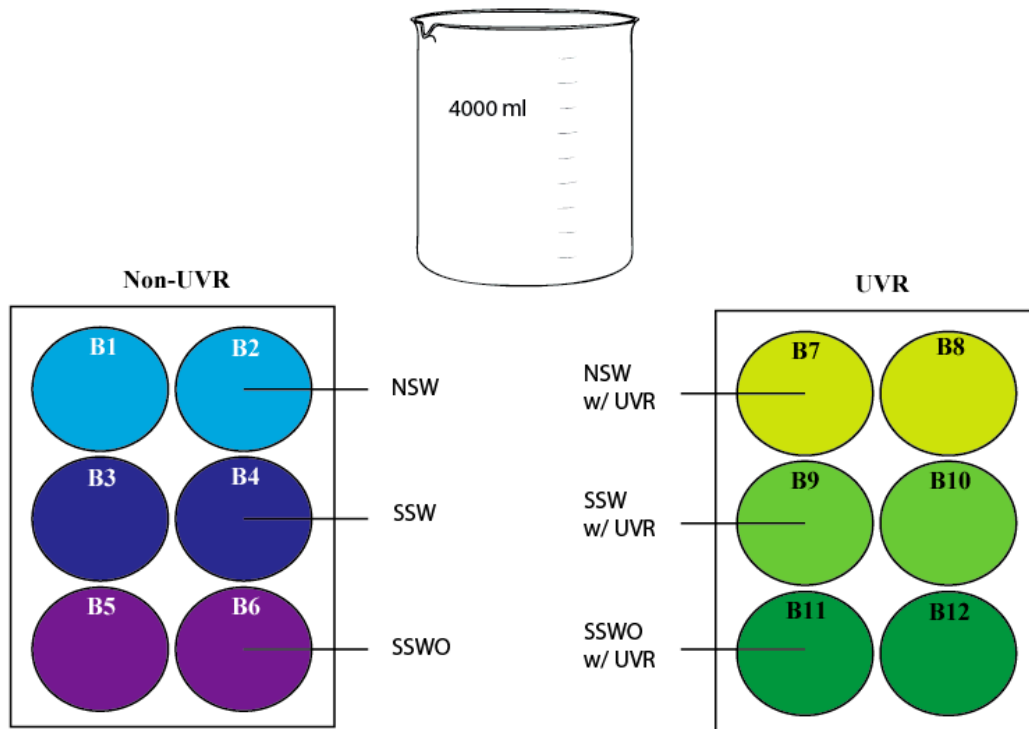


Figure 3. Setup in the survival experiment. Beakers 1-6 were not exposed to UVR, while beakers 7-12 were. NSW: natural seawater (B1, B2), SSW: sterile seawater (B3, B4), SSWO: sterile seawater and oil (B5, B6), NSW w/UVR: Natural seawater exposed to ultra violet radiation (UVR) (B7, B8), SSW w/UVR: sterile seawater exposed to UVR (B9, B10), SSWO w/UVR: sterile seawater and oil exposed to UVR (B11, B12).

To each of the 12 beakers, 22.5 mL of sterile filtered supernatant, with a titre of approximately 1.58×10^6 (TCID₅₀) virus per mL, containing the SAV 3 isolate YK230812-7, were added. In the 4 beakers containing an oil layer, the 22.5 mL of supernatant was added to the 115 mL of oil before the mixture then was added to the respective beakers.

UV dose

Measured UV index-values from light tubes yielded a value of 22 at the surface of the water in the beakers, and a mean value of 11 in the beakers, given an effective attenuation coefficient of $K_d=8$. The mean UV index in Bergen (60° North and 5° East) at noon on a sunny day during the summer is approximately 5 (<http://www.nrpa.no/uvnett/dagsverdi.asp>). UV-A (320-400 nm) and UV-B (280-320 nm) radiation were used in the experiment, provided by six UV-A Philips TL20W/05 and five UV-B Philips TL20W/12 fluorescent light tubes. The UV spectrum used in the experiment can be seen in Figure 4.

Wavelengths in the UV-C spectrum (200-280 nm) from the sun do not reach the earth's surface (Diffey, 1991), and were therefore not included in the experiment.

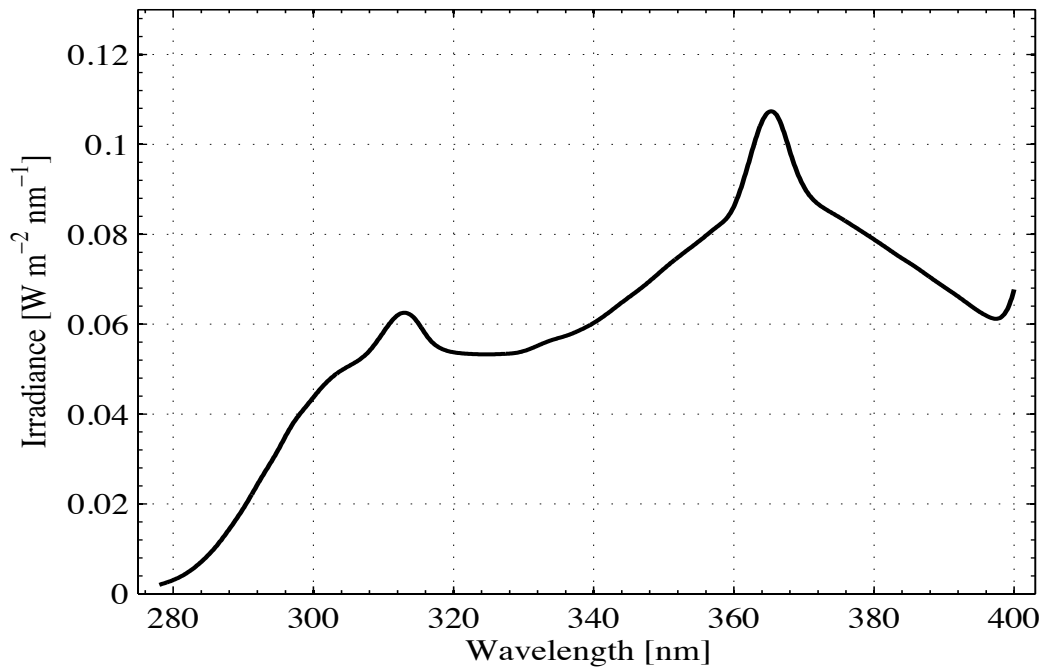


Figure 4. Measured UV irradiances, in the UV-B (280-320 nm) and UV-A (320-400 nm) part of the spectrum, used in the experiment.

A total of 10 samples were taken from each beaker, with sampling points at following times; at start, after 3 hours, 6 hours, 9 hours, 12 hours, 18 hours, 24 hours, 36 hours, 48 hours and 72 hours, which are named T_0 - T_{72} , respectively. For each sampling point, two samples of 50 mL were taken from each beaker, one for cell culture and one for real-time RT-PCR, making the total of samples per sampling point 26, including the two negative filtration controls containing Milli-Q® H₂O (distilled water).

Water filtration

The water samples were filtered through an electropositive Zeta Plus® Virosorb® 1 MDS filter (Cuno Inc., U.S.A.) to isolate the viral particles from the sample according to a VIRADEL (virus-absorption-elution) method described by Andersen et al. (2010).

Water samples for cell culture

13 of the filtered water samples, one from each beaker (1-12) plus a negative control containing dH₂O, were subsequently put on ice in a petri dish containing 1.4 ml EMEM with 2 % FBS. After approximately 10 minutes on a tilt tray, 350 µL were transferred to CHSE-214 cells grown in 25-cm² Nunc™ cell flask containing 4.5 ml 2 % EMEM. Cell culture flasks were incubated at 14.5 °C. After three weeks, the supernatant was sterile filtered and passed to cell culture dishes in duplicates. One replicate was used for immunofluorescence test as described under IFAT (Figure 5), and from the other, RNA was extracted and analysed by real-time RT-PCR (see Tab. 9 under results).

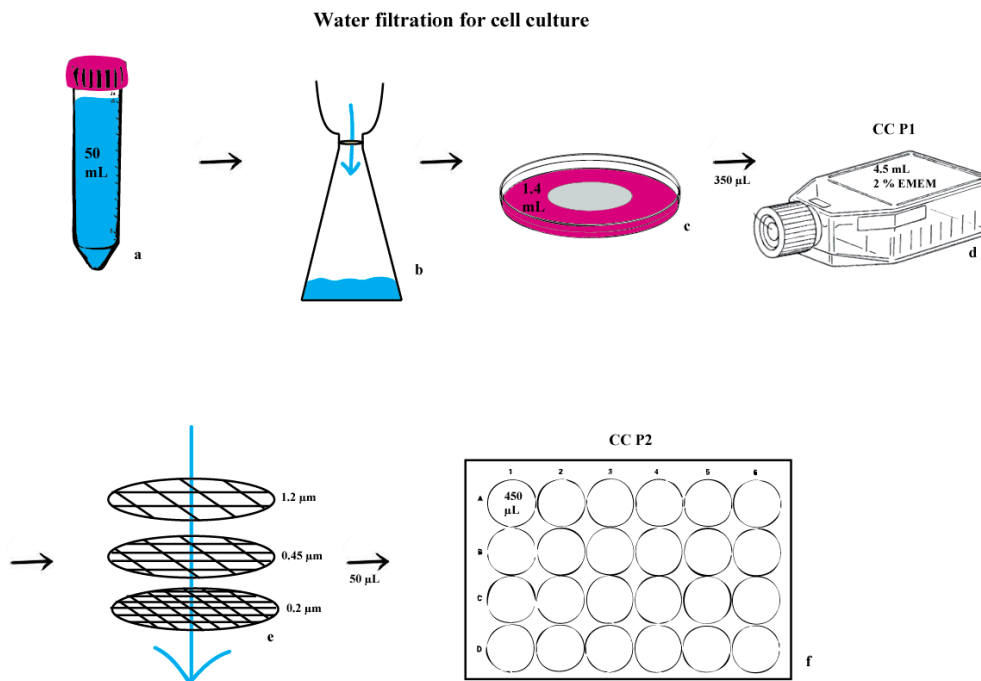


Figure 5. Passages of filtered virions from water samples in cell cultures. 50 mL water sample (a) was filtered through an electropositive filter (b). Absorbed virions were released in cell medium (c). Cell cultures (CC P1) passage 1 were incubated for 21 days at 14.5 °C in 25 cm² Nunc flasks with 350 µL inoculum added to 4.5 mL 2 % EMEM (d). Between CC P1 and P2, the supernatant was sterile filtered (e) and 50 µL was inoculated in 450 µL 2 % EMEM (f) in cell culture dishes.

Water samples for real-time RT-PCR

The 13 filters from the water samples for real-time RT-PCR were put in a petri dish containing 1.4 mL TRK lysis buffer (E.Z.N.A total RNA kit from Omega BioTek®). After 10 minutes on ice, 350 µL was transferred to a 1.5 mL Eppendorf™ column, and subsequently 350 µL 70 % Ethanol was added, giving a total volume of 700 µL.

Both an A and B sample was taken. The samples were then stored at -80 °C. 10 µL of ISAV supernatant containing the ISA virus isolate CH35/09 (Accession nr: KC905164) were added to the water samples prior to filtration as an exogenous filtration control, i.e. a reference for normalizing real-time RT-PCR Ct-values. As it turned out, the added spike (exogenous control) was, in many of the samples, not detectable by real-time RT-PCR. Thus, the results from A samples were not usable, and B samples were thawed and RNA was extracted from them. All B samples were therefore added another 100 µL (Ct-value \approx 26.5) of the spike prior to RNA extraction.

Normalization was used for relative quantification of Ct-values obtained in the experiment. Normalized expressions (NE) were calculated using the formula

$$NE = \frac{(E_{target})^{Ct_{target}}}{(E_{ref})^{Ct_{ref}}}$$

from Muller et al. (2002), where the NSAV assay was used as *target* and the ISAV7 assay was used as *ref*. All mean normalized expression values were transformed into NE-fold and log2 as described by Andersen et al. (2010).

Real-time RT-PCR

All real-time RT-PCR analyses were run in a 7500 Real-Time PCR System (Life Technologies™) according to standard AgPath-ID one-step RT-PCR kit (Life Technologies™) protocol, with a few exceptions. 6.25 µL of 2X RT-buffer and 0.5 µL enzyme mix were used for all the three assays, with the primer and probe varying in concentration. All the three assays used, listed in Table 1, were optimized in regard to primer and probe concentration against the AgPath-ID one-step RT-PCR kit. A total amount of 12.5 µL in each well was used, which is half of the standard AgPath kit. The reactions were run according to Standard AgPath setup, which is 45 °C for 10 minutes, 95 °C for 10 minutes and then 45 cycles with 95 °C for 15 seconds and 60 °C for 45 seconds. RNA extraction control (NC) and non-template control (NTC) were included with every run.

Table 2. Assays used for real-time RT-PCR. nsP1 assay targeting the nsP1 gene present in all SAV subtypes, NSAV assay targeting only SAV 3 and ISAV S7 targeting segment 7 in ISAV.

Name	Sequence	Source
Q_nsP1 F	5'-CCGGCCCTGAACCAGTT-3'	(Hodneland and Endresen, 2006)
Q_nsP1 R	5'-GTAGCCAAGTGGGAGAAAGCT-3'	
nsP1 probe	FAM-5'-TCGAAGTGGTGGCCAG-3'-MGB	
Q_NS AV F	5'-CAGTCAAATTCGATAAGAAGTGCAA-3'	(Hodneland and Endresen, 2006)
Q_NS AV R	5'-TGGGAGTCGCTGGTAAAGGT-3'	
NSAV probe	FAM-5'-AGCGCTGCCCAAGCGACCG-3'-MGB	
ISAV S7-3F	5'-TGGGATCATGTGTTTCCTGCTA-3'	(Plarre et al., 2005)
ISAV S7-3R	5'-GAAAATCCATGTTCTCAGATGCAA-3'	
ISAV S7-probe	FAM-5'-CACATGACCCCTCGTC-3'-MGB	

Optimization

For all the three assays, different primer concentrations were tested and Ct-values registered. The optimal concentration, using the same amount of template, was then identified by analysing the amplification and multicomponent plot. The following concentrations were tested (Table 3).

Table 3. Optimization of forward and reverse primer concentrations against AgPath-ID™ One-step Kit.

Forward/Reverse	F primer (200 nM)	F primer (400 nM)	F primer (600 nM)	F primer (800 nM)
R primer (200 nM)	200/200	400/200	600/200	800/200
R primer (400 nM)	200/400	400/400	600/400	800/400
R primer (600 nM)	200/600	400/600	600/600	800/600
R primer (800 nM)	200/800	400/800	600/800	800/800

After the optimal primer concentration was determined, it was tested against different probe concentrations to determine optimal probe concentration. The following concentrations were tested (Table 4).

Table 4. Optimization of probe concentrations against AgPath-ID™ One-step Kit.

nM	nM	nM	nM	nM	nM	nM
45	70	95	120	145	170	195

Efficiency test

To test the three assays ability to detect their respective target template, an efficiency test was performed. A serial tenfold dilution of known template was made and analysed with real-time RT-PCR. The dilution series were run in triplicates, and the mean Ct-value was plotted against the dilution series in Microsoft Excel where slope and regression for the graphs were calculated. The efficiency was then calculated using the formula: $E = 10^{(-1/slope)}$. If the assay has 100 % efficiency, the test should result in a standard curve with a slope of -3.322, which means that the amount of PCR product is doubled per cycle.

Extraction of RNA

In order to perform real-time RT-PCR, total RNA from samples was extracted using the E.Z.N.A Total RNA kit I (Omega Bio-Tek®) according to standard protocol from the manufacturer. RNA from cells from passage two after filtration (CC P2) was extracted according to the method described by Devold et al. (2001).

Indirect fluorescence antibody test (IFAT)

To provide evidence of replication in all cultures, indirect fluorescence antibody test was performed on cells grown on coverslips in 24-well culture dishes. 50 µL of sterile filtered supernatant from the cell culture flasks from the first passage after water filtration (see Fig. 5) was used as inoculum. Each well contained a 60-80 % confluent monolayer of CHSE cells and 450 µL of 2 % EMEM. 12 uninfected wells were used as negative controls. Following an incubation of 14 days on 14.5 °C, IFAT was performed as described by Karlsen et al. (2010) with one exception. Secondary antibody Alexa Fluor® 488 donkey anti-rabbit IgG (Life Technologies™) was diluted 1:200 in PBS-FBS with 5 % skimmed milk. In order to perform the IFA test, primary, polyclonal antibodies, E2-pTE200 Final bleed antisera 90393 Rabbit 2443, were absorbed against CHSE cells and subsequently diluted 1:10 in 1 x PBS pH 7.33 according to the method described by Karlsen et al. (2010).

After incubation with secondary antibodies, the coverslips were washed three times in 1x PBS, carefully dried and placed on a slide containing 5 µL of ProLong®

Gold Antifade Reagent with DAPI (Life Technologies™). The preparations were stored in the dark at room temperature over night before being transferred to 4 °C.

Because the fixation process was suboptimal, the results from the first IFA test were not usable. Hence, the test was performed a second time. Supernatant from the first passage after filtration (CC P1) was thawed and once again used as inoculum. The rest of the process was identical to the first time.

Endpoint titration

Virus end point-titration of the supernatant added in the survival experiment was performed as described by Andersen et al. (2010), only that CHSE-214 cells were used instead of RT-gill cells. IFAT was performed as previously described, and titre was calculated after the method of Karber (1931).

Results

Pilot study

During the pilot study SAV RNA was detected by real-time RT-PCR from cell culture, sterile filtered supernatant from the same cell culture (inoculum used in the study), and from cell culture infected with obtained virions from water samples ($T_{75 \text{ min}}$ - $T_{48 \text{ t}}$) (Table 5).

Table 5. Real-time RT-PCR Ct-values from pilot study. The assay used is nsP1. $T_{75 \text{ min}}$ - $T_{48 \text{ t}}$ are Ct-values from 100 μL of supernatant from infected cell cultures with virions obtained from water samples at each sampling point. SAV YK230812-7 st. sup. is 100 μL of the inoculum used and CHSE YK230812-7 is RNA extracted from cells infected with SAV.

Sample	Ct-value
$T_{75 \text{ min}}$	28.5
$T_{195 \text{ min}}$	29.0
$T_{24 \text{ t}}$	28.9
$T_{48 \text{ t}}$	29.8
SAV YK230812-7 st. sup.	22.6
CHSE YK230812-7	13.3

Optimization of primers and probe

The different assays were optimized against AgPath-ID™ One-step RT-PCR Kit (Life technologies™) regarding the optimal primer and probe concentration.

Table 6. Optimized primer and probe concentration using AgPath-ID™ One-step RT-PCR Kit.

	nsP1	NSAV	ISAV7
F primer	800 nM	200 nM	600 nM
R primer	800 nM	600 nM	200 nM
Probe	195 nM	195 nM	195 nM

An efficiency plot was also drawn for all the assays as seen in Figure 6. The same template was used for both SAV assays.

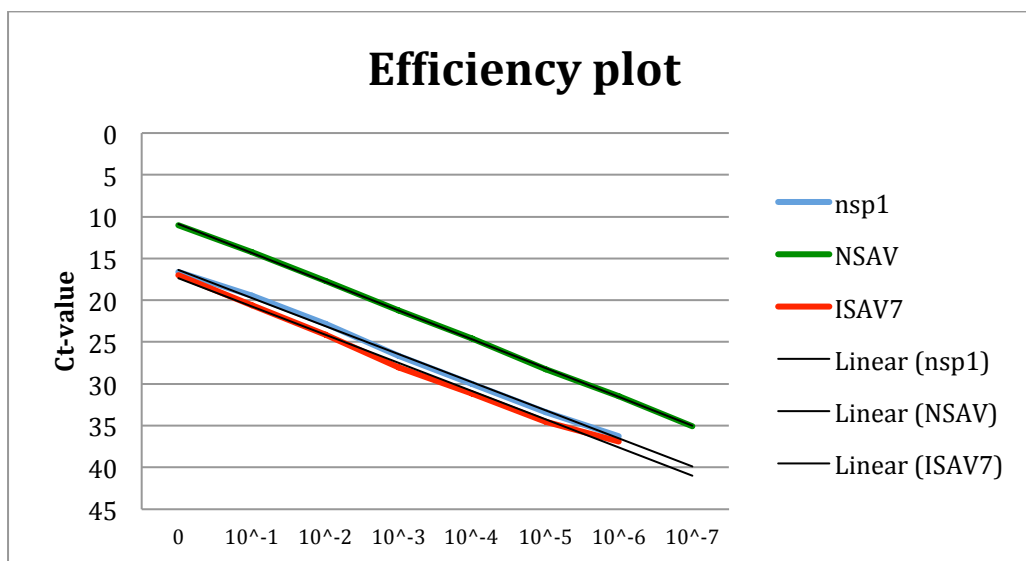


Figure 6. Standard curve for nsP1, NSAV and ISAV7 assays.

Table 7. Calculated values for slope, regression (R^2) and efficiency (E) for nsP1, NSAV and ISAV7 assays.

Assay	Slope	R^2	E
nsP1	3,3583	0,99873	1,9850
NSAV	3,4457	0,99985	1,9508
ISAV7	3,3746	0,99652	1,9785

The survival experiment

The water temperature in the beakers ranged from 7 °C to 11 °C during the experiment. The temperature was registered at every sampling time. All registrations can be seen in Figure 7.

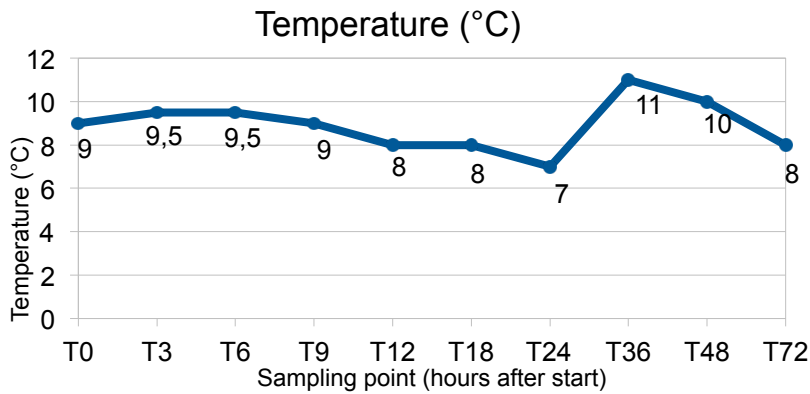


Figure 7. Temperature regime during the survival experiment.

Detection of SAV RNA in water samples

All real-time RT-PCR analyses were positive for SAV RNA throughout the whole experiment, except one sample (sterile seawater exposed to UVR in beaker 9 at T₇₂) when using the nsP1 assay. Figures 8-13 show the normalized Ct-values plotted against time (sampling point), using the NSAV assay for SAV and the ISAV7 assay for the exogenous control (ISAV). All mean normalized expression (MNE) values were transformed into NE-fold by defining the lowest MNE value obtained in the experiment, which was the sample from beaker 10 at T₇₂. Both A and B samples were analysed in this study, but only the results from the B samples are presented here.

The water samples from all beakers not exposed to UVR (Figure 8, 10, 12) contained approximately the same amount SAV RNA throughout the experiment, although some fluctuations in the amount of RNA were observed. However, the relative amount of viral RNA was higher in the two beakers containing an oil layer (Figure 12). Presence of SAV RNA in the beakers exposed to UVR (Figure 9, 11, 13) showed a steady decline throughout the experiment. In the beakers containing an oil layer (Figure 13), the relative amount of SAV RNA was higher, but the same trend could be seen during the experiment. All the extraction (NC) and non-template (NTC) controls were negative.

Natural seawater



Figure 8. Mean normalized expression values of SAV RNA from beaker 1 and 2 with natural seawater.

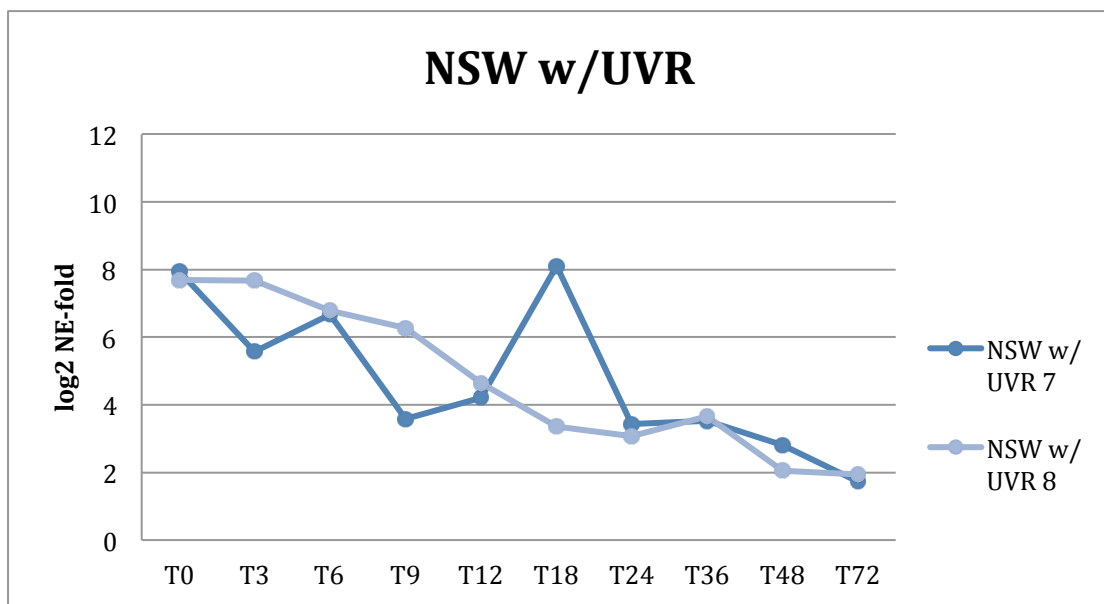


Figure 9. Mean normalized expression values of SAV RNA from beaker 7 and 8 with natural seawater exposed to UVR.

Sterile seawater

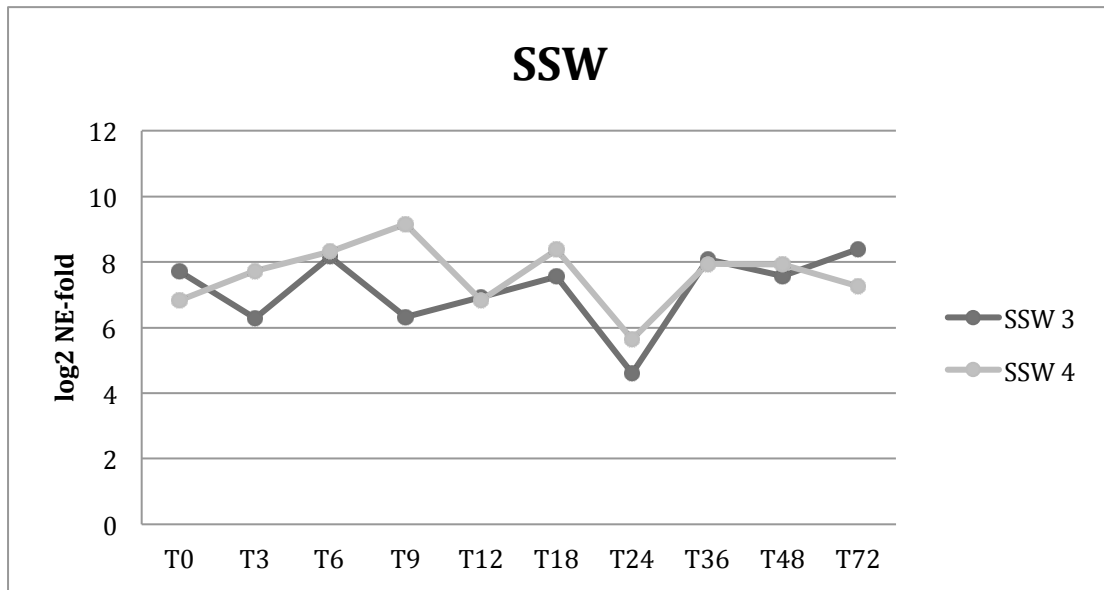


Figure 10. Mean normalized expression values of SAV RNA from beaker 3 and 4 with sterile seawater.

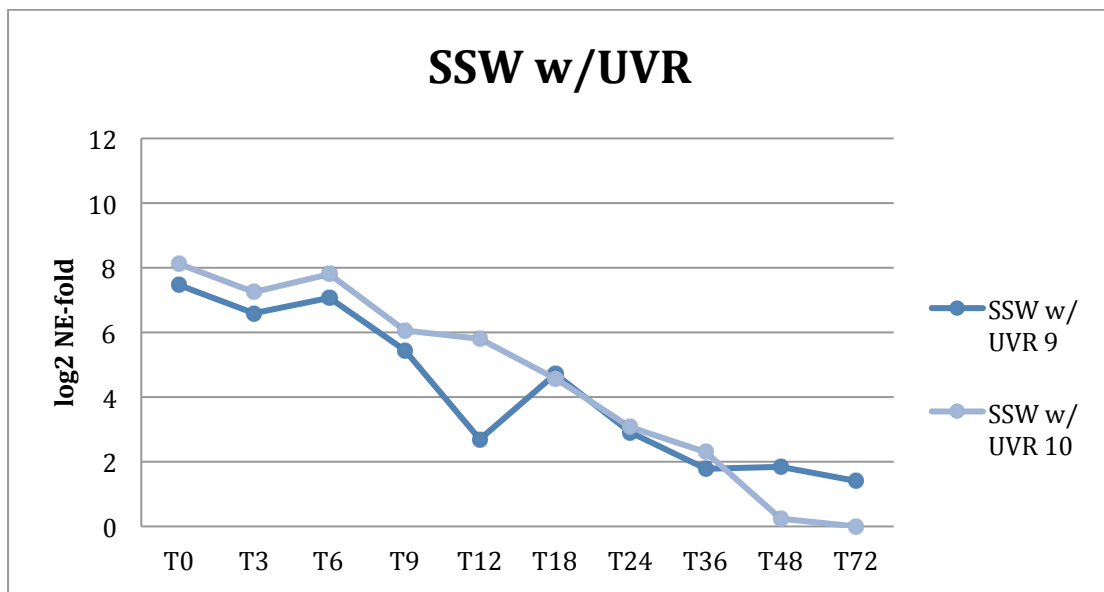


Figure 11. Mean normalized expression values of SAV RNA from beaker 9 and 10 with sterile seawater exposed to UVR.

Sterile seawater and oil

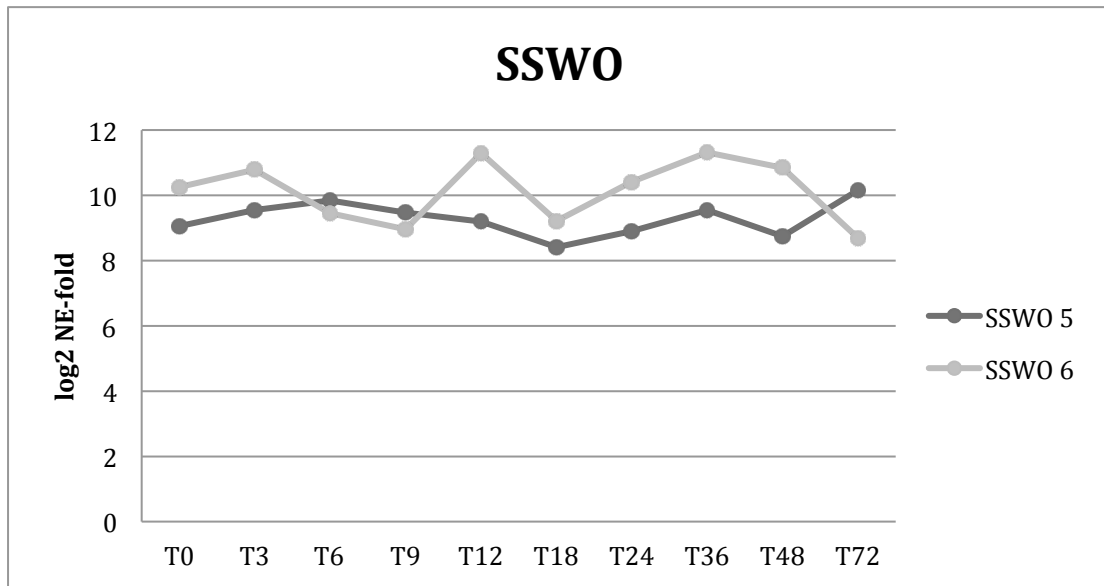


Figure 12. Mean normalized expression values of SAV RNA from beaker 5 and 6 with sterile seawater and oil.

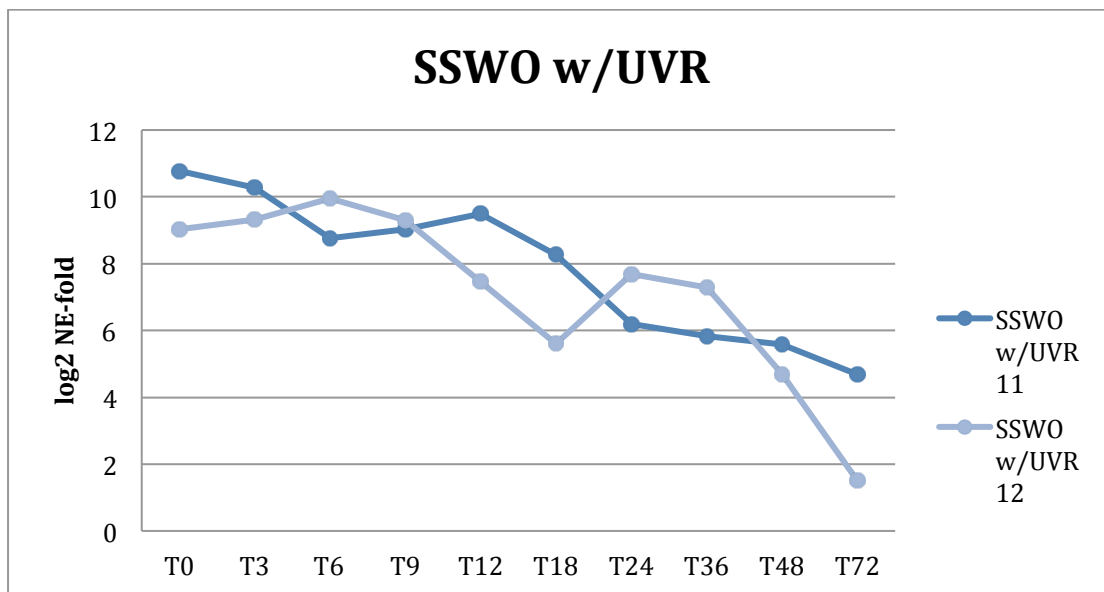


Figure 13. Mean normalized expression values of SAV RNA from beaker 11 and 12 with sterile seawater and oil exposed to UVR.

SAV infectivity in cell culture

Indirect fluorescence antibody test

As seen in Table 8., infective virions were obtained from water samples throughout the experiment in beakers 3-6 (SSW and SSWO). For the beakers with natural seawater (NSW), infective virions were obtained 48 h after start in beaker 1, while infectivity was lost between 24 and 36 hours in beaker 2. In beakers exposed to UVR containing natural and sterile seawater (NSW w/UVR and SSW w/UVR) no viable virions could be obtained three hours after start. In SSWO w/UVR viable virions were obtained three hours after start, but not after six hours and throughout the rest of the experiment.

Natural seawater (NSW)

In none of the beakers containing untreated, non-sterile seawater viable virions were obtained throughout the whole experiment. In beaker 1, infectivity was lost between 48-72 h, and in beaker 2 between 24-36 h.

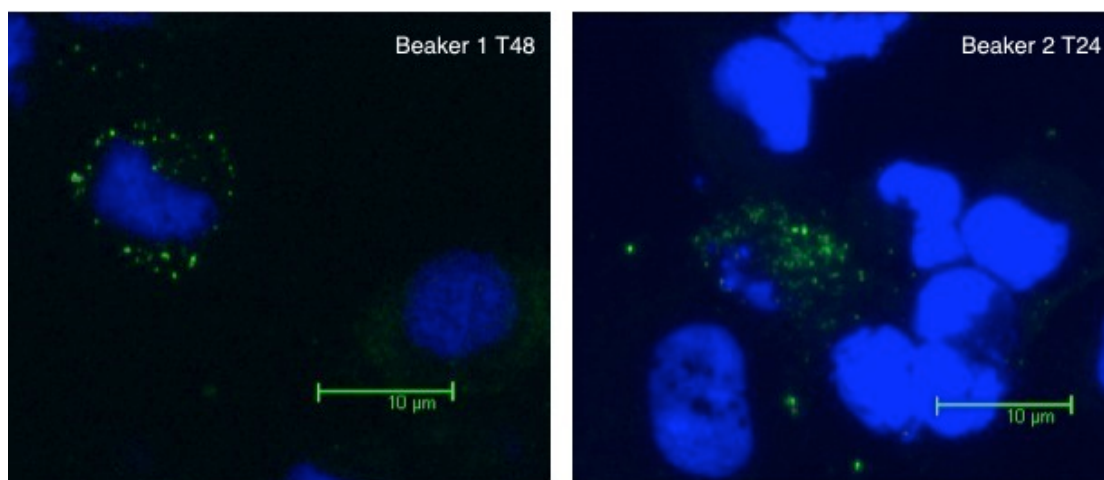


Figure 14. IFAT performed on cells infected with supernatant from the first passage after filtration. The picture to the left shows an infected cell from the cell culture inoculated with the water sample from beaker 1 after 48 hours, while the picture to the right shows an infected cell inoculated with the water sample from beaker 2 after 24 hours. Both pictures are from the last positive sample from beaker 1 and 2, respectively.

Sterile seawater (SSW)

In the beakers containing sterile seawater, viable virions were obtained throughout the experiment.

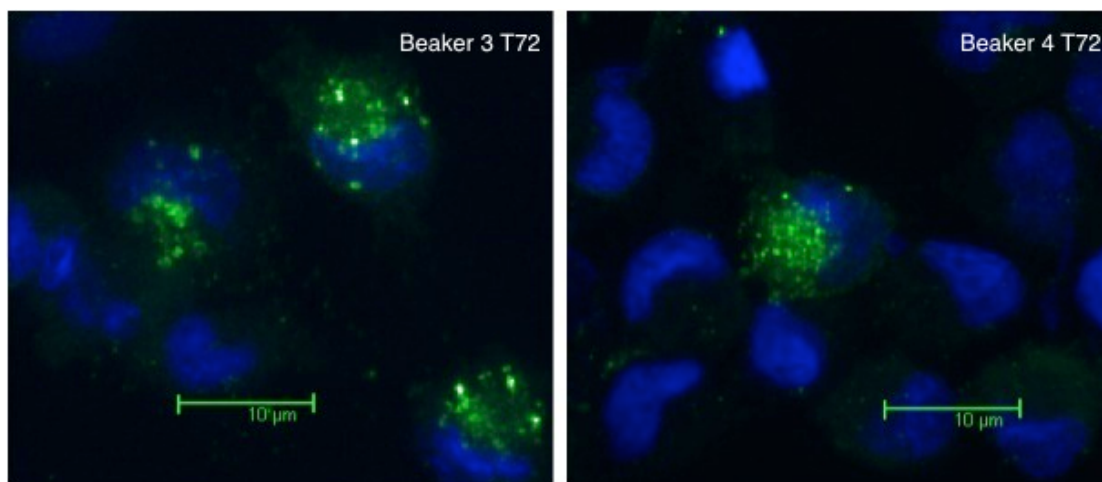


Figure 15. IFAT performed on cells infected with supernatant from the first passage after filtration. The picture to the left shows infected cells from the cell culture inoculated with the water sample from beaker 3 after 72 hours, while the picture to the right shows an infected cell inoculated with the water sample from beaker 4 after 72 hours. Both pictures are from the last sampling time (T72).

Sterile seawater and oil (SSWO)

Viable virions were obtained from beakers 5 and 6 at all sampling times during the experiment.

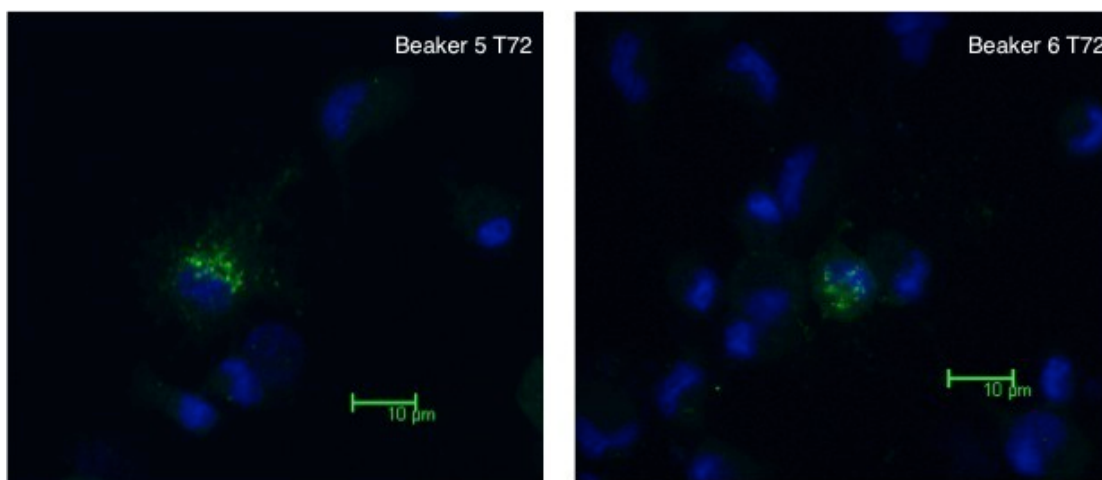


Figure 16. IFAT performed on cells infected with supernatant from the first passage after filtration. The picture to the left shows an infected cell from the cell culture inoculated with the water sample from beaker 5 after 72 hours, while the picture to the right shows an infected cell inoculated with the water sample from beaker 6 after 72 hours. Both pictures are from the last sampling time (T72).

Natural seawater with UVR (NSW w/UVR)

In beaker 7, no viable virions were obtained after three hours and throughout the experiment. In beaker 8, no viable virions were obtained at all.

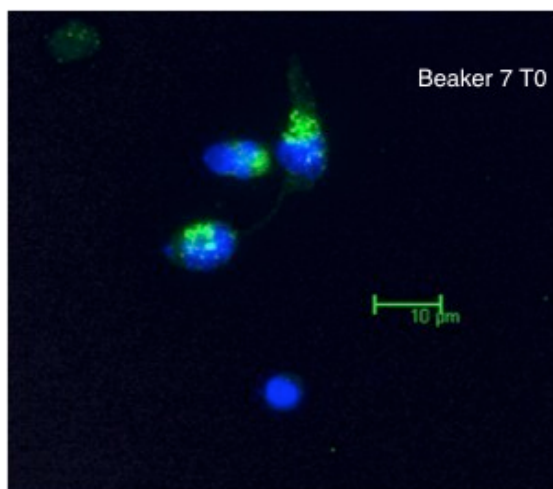


Figure 17. IFAT performed on cells infected with supernatant from the first passage after filtration. The picture shows infected cells from the cell culture inoculated with the water sample from beaker 7 at start (T0), which was the only positive sample from this beaker.

Sterile seawater with UVR (SSW w/UVR)

In these two beakers, viable virions were only obtained at the start of the experiment.

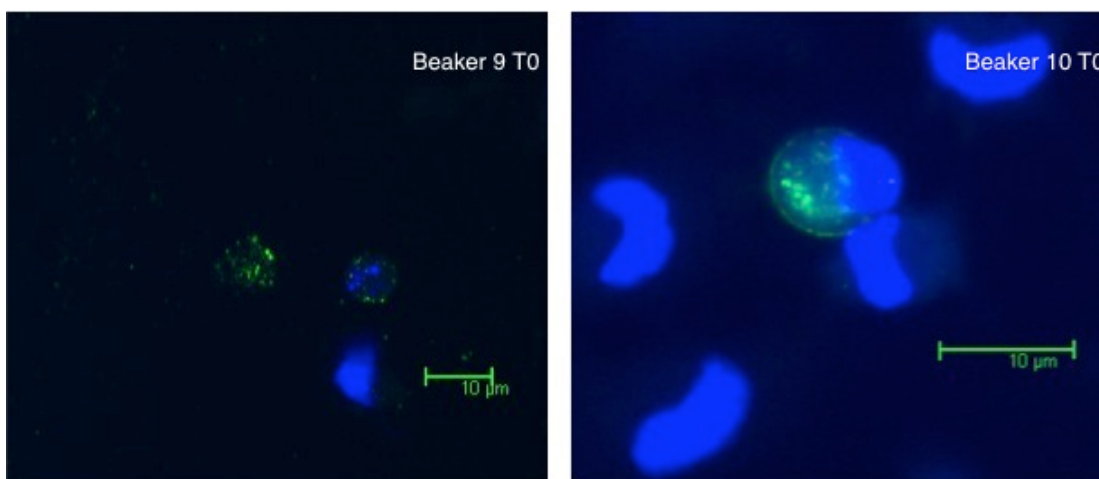


Figure 18. IFAT performed on cells infected with supernatant from the first passage after filtration. The picture to the left shows an infected cell from the cell culture inoculated with the water sample from beaker 9 at start, while the picture to the right shows an infected cell from the cell culture inoculated with the water sample from beaker 10 at start. These were the only positive samples from beaker 9 and 10, respectively.

Sterile seawater and oil with UVR (SSWO w/UVR)

In beaker 11 and 12, viable virions were obtained both at T₀ and at T₃. From T₆ and throughout the experiment, no viable virions were obtained.

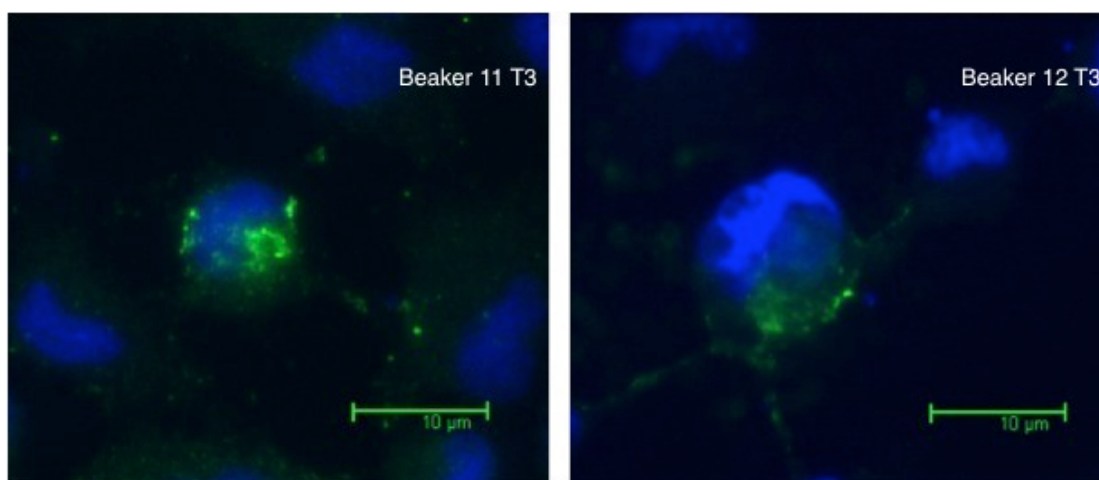


Figure 19. IFAT performed on cells infected with supernatant from the first passage after filtration. The picture to the left shows an infected cell from the cell culture inoculated with the water sample from beaker 11 after 3 hours, while the picture to the right shows an infected cell from the cell culture inoculated with the water sample from beaker 12 after 3 hours. Both pictures are from the last positive sample from beaker 11 and 12, respectively.

A full view of IFAT results can be seen in Table 8. The results are based on subjective assessments.

Table 8. Results of indirect fluorescence antibody test where 1 indicates a positive sample and 0 indicates a negative sample. 1/0 indicates a weak positive/undetermined. Beaker 1 & 2: natural seawater (NSW), beaker 3 & 4: sterile seawater (SSW), beaker 5 & 6: sterile seawater and oil (SSWO), beaker 7 & 8: natural seawater with UVR (NSW w/UVR), beaker 9 & 10: sterile seawater with UVR (SSW w/UVR), beaker 11 & 12: sterile seawater and oil with UVR (SSWO w/UVR).

Beaker	T0	T3	T6	T9	T12	T18	T24	T36	T48	T72
1	1	1	1	1	1	1	1	1	1/0	0
2	1	1	1	1	1	1	1/0	0	0	0
3	1	1	1/0	1	1	1	1/0	1/0	1	1/0
4	1/0	1/0	1	1	1	1	1	1	1/0	1
5	1	1	1	1	1	1	1/0	1	1	1/0
6	1	1/0	1	1/0	1	1	1	1	1	1
7	1	0	0	0	0	0	0	0	0	0
8	1/0	0	0	0	0	0	0	0	0	0
9	1/0	0	0	0	0	0	0	0	0	0
10	1	0	0	0	0	0	0	0	0	0
11	1	1	0	0	0	0	0	0	0	0
12	1/0	1	0	0	0	0	0	0	0	0

Real-time RT-PCR of infected cells

Real-time RT-PCR from infected cell cultures yielded significantly lower Ct-values (Ct-values 20.2-36.5; mean: 24.5) than RNA isolated directly from water samples (26.6-36.9; mean: 31.5), which strongly suggest that replication has occurred.

Table 9. Real-time RT-PCR Ct-values of RNA extracted from cell culture P2 infected with sterile filtered supernatant from cell culture P1, i.e. the first passage after water filtration. The assay used is nsP1. (UD = undetermined)

Beaker	T0	T3	T6	T9	T12	T18	T24	T36	T48	T72
1	20,4	25,4	23,5	21,2	22,4	22,1	20,2	23,6	35,8	UD
2	21,9	23,7	21,6	21,7	22,9	23,2	27,8	UD	UD	UD
3	22,5	23,6	22,5	23,3	22,3	25,5	23,3	23,4	32,2	27,3
4	22,2	36,5	23,4	24,4	23,8	24,2	25,9	24,8	33,2	27,9
5	21,7	22,2	22,5	23,2	23,8	23,9	32,3	28	UD	28,3
6	21,2	20,3	22,8	23,2	23,8	22,9	25,1	28,2	36,5	26,3
7	20,8	UD	UD	UD	UD	UD	UD	UD	UD	UD
8	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
9	22,5	UD	UD	UD	UD	UD	UD	UD	UD	UD
10	21,4	UD	UD	UD	UD	UD	UD	UD	UD	UD
11	22,7	22,2	UD	UD	UD	UD	UD	UD	UD	UD
12	22,3	20,7	UD	UD	UD	UD	UD	UD	UD	UD

Discussion

Pancreas disease in Norwegian aquaculture was earlier confined to farms in the Hordaland County on the western coast of Norway (Karlsen et al., 2006). However, since 2003, an increasing number of PD outbreaks have occurred both north and south of the initial enzootic zone and the geographical distribution of SAV 3 now includes all of western Norway, south of Hustadvika in Møre and Romsdal County. Ever since SAV 2 was introduced to Norwegian aquaculture in 2010, a similar pattern has been seen with this SAV subtype north of the enzootic zone for SAV 3. This pattern indicates that horizontal transmission of the virus could be the main pathway of infecting new cohorts.

Over the last decade hydrodynamic models have been used to explain spreading patterns of several fish pathogens (e.g. Murray and Gillibrand, 2006, Gustafson et al., 2007, Amundrud and Murray, 2009), and two studies have found waterborne transmission of SAV by ocean currents to explain the pattern for outbreaks of PD in two separate Norwegian fjords by using these models (Viljugrein et al., 2009, Stene et al., 2014). One important premise in such models is the survival of the free virions in seawater, as survival times limits the potential to infect new cohorts. There is, however, only one previous study on SAV survival in seawater, which is referred to in the studies above. Graham et al. (2007) reported that SAV can remain infective >63 days in sterile seawater at ≤ 10 °C, and although this may provide the upper limit of SAV survival, which can be helpful in risk assessment for PD, it does not account for several virucidal factors present in the natural environment.

Many studies have stated that virucidal microorganisms, heavy metals, non-living particles and sunlight, all can have devastating effects on viral survival or infectivity in seawater (e.g. Murray and Jackson, 1993). Several experimental studies have confirmed that viral survival is negatively related to bacterial biomass in the water (Herrmann et al., 1974, Berry and Noton, 1976, Fujioka et al., 1980, Toranzo and Metricic, 1982, Suttle and Chen, 1992, Murray and Jackson, 1992, Brady et al., 1993), which is consistent with the results in the current study where survival was reduced in beakers with natural seawater (<72 hours) as opposed to sterile seawater (>72 hours). Graham et al. (2007) also reported markedly reduced survival for SAV in non-sterile seawater when compared to sterile conditions. However, in the study by

Graham et al. (2007), viable virions were obtained after 14 days at 10 °C and 35 days at 4 °C in non-sterile seawater. Infectivity was lost somewhere within the first 7 days at both 15 °C and 20 °C, as no viable virions were ever obtained after the experiment had started (Graham et al., 2007). In the current study, the last infective virions were obtained after 24 and 48 hours in the two beakers containing non-sterile seawater, and although Graham et al. (2007) used isolates belonging to SAV subtype 1, and an isolate belonging to the SAV subtype 3 was used in this study, it is not anticipated that there are any biophysical differences between the different SAV subtypes regarding survival in seawater, given that they both represent one serotype (Christie et al., 1998, McLoughlin et al., 1998, Graham et al., 2003). In the study by Viljugrein et al. (2009) mentioned above, Graham et al. (2007) are cited regarding survival times for SAV, but they do not state what survival times they operate with in the hydrodynamic model. In the study by Stene et al. (2014), assumed survival times were stated to be a 50 % reduction in viral titre of 4.3 days in the winter and 1 day in the summer, consistent with survival times from Graham et al. (2007) in non-sterile seawater at 4 °C and 10 °C, respectively. However, based on results in non-sterile seawater in the current study, SAV survival is significantly shorter, and hence, infective virions will not have the same potential to reach cohorts in adjacent farms by passive drift by ocean currents as assumed in the models mentioned above.

Nevertheless, waterborne, horizontal transmission by passive drift by ocean currents can not explain all aspects of transmission of SAV in aquaculture as sporadic outbreaks of PD occur in the northern counties of Norway. This phenomenon has earlier been associated with well-boat transportation from the enzootic zone on the western coast of Norway (Karlsen et al., 2006). Whether this implies that the fish were carriers of SAV from the fresh water phase or were infected during the transport is not known. However, the results in the current study confirm that the fish could potentially be infected by SAV when passing by infected sites in a viraemic phase during open well-boat transport.

Many authors have interpreted the observation that PD outbreaks have a tendency to cluster in time and space as an indication for waterborne transmission between adjacent farms (e.g. Kristoffersen et al., 2009). However, Karlsen et al. (2014) have reported findings of genetic distinct strains of SAV 3 in the same time and space, and genetic identical strains separated by large geographical distances, indicating that waterborne, horizontal transmission may not contribute to

dissemination of the virus as much as considered by many authors. Based on findings of SAV in marine flat fish in close proximity to salmon farms (Snow, 2011, Bruno et al., 2014, McCleary et al., 2014), and the genetic diversity of SAV within a geographical area could indicate a marine reservoir with continuous transmission between farmed and wild fish (Karlsen et al., 2014).

The exact endpoint of SAV infectivity is not known for either Graham et al. (2007) or the current study, but as the temperature in this experiment was lower than 10 °C the majority of the time, survival times in the natural seawater was, based on Graham et al. (2007)'s findings, expected to last throughout the experiment. However, an obvious concern, or weakness, regarding experiments under non-sterile conditions is that data from different studies are not directly comparable, nor is it possible to reproduce identical conditions in future experiments. In this study the non-sterile seawater was collected at daytime in late October in surface waters in the vicinity of several salmon farms. It is conceivable that biological activity in seawater will vary during the day, year, with depth and with geographical position, but the water used in this study is assumed to represent natural conditions for waterborne SAV particles in Norwegian coastal waters, and the non-sterile seawater used in the study by Graham et al. (2007), which was also collected in the vicinity of a salmon farm, may have had different concentrations of heavy metals, bacteria and protozoa, hence leading to longer survival times.

In this study, the sterile seawater was not filtered to remove any particulate organic material (POM), which is, with a few exceptions, known to increase viral decay in water (Wommack and Colwell, 2000). However, autoclaving the water has been shown to have the same effect on antiviral activity as filtration through 0.2 µm filters (Berry and Noton, 1976, Fujioka et al., 1980, Wommack and Colwell, 2000), and the results from the present experiment indicate that virucidal activity was indeed reduced in the autoclaved seawater, as infective virions were re-isolated after 72 hours, as opposed to 24-48 hours in non-sterile seawater. Graham et al. (2007) also reported reduced survival times in sterile seawater with the presence of organic matter. However, when exposed to UV and given high enough concentrations of POM, it may have an enhancing effect on survival, protecting virus from degrading by absorbing UV-B light (Suttle and Chen, 1992). An enhancing effect by presence of POM on viral survival could not be determined in the current study, as filtered control water was not included.

Viral titres may be as high as 10^{10} TCID₅₀/50 µL in serum of infected salmon during viraemia (Jewhurst et al., 2004). A substantial amount of virus could therefore be shedded from an infected farm during an outbreak, as SAV RNA has been detected in the water during viraemia in a challenge experiment, where quantity of viral RNA in the water followed the same pattern as viral load measured in plasma (Andersen et al., 2010). A recent study also found infective SAV particles in mucus and faeces from PD diseased salmon, indicating that shedding of virus may happen from the skin and gut (Graham et al., 2012). Jansen et al. (2010b) found that once SAV was detected by real-time RT-PCR at a site, all sampled individuals tested positive throughout the production cycle. Together with results from the current study and Graham et al. (2007), this strongly suggests waterborne, horizontal transmission between individuals and between the cages at a site. However, regarding waterborne transmission between adjacent farms, results are not conclusive. The concentration of free, waterborne virions will be diluted in the water column, and the chance of an infective dose of SAV virions reaching other farms is uncertain.

In a similar experiment to the current study, conducted by Vike et al. (2014), it was shown that ISAV virions were still infective after 12 hours in sterile seawater. However, in untreated seawater, infectivity was lost within the first three hours. This is significantly shorter than survival times for SAV in natural seawater (<72) presented in the current study, indicating that SAV is somewhat more robust than ISAV. Consequently, waterborne transmission of SAV seems to be a more likely transmission pathway than for ISAV. Regarding differences in UVR resistance between SAV and ISAV, data are not conclusive, as, like the results from the current study, viral infectivity was lost in beakers with natural and sterile seawater exposed to UVR during the first 3 hours.

A lipid film on the sea surface, due to lipids hydrophobic nature and positive buoyancy in water, is often seen around salmon farms, probably originating from feed pellets. However, during disease outbreaks associated with mortality, this lipid film seems to be more prominent, likely due to lipid droplets released from dead, decaying salmon in the cages (Stene et al., 2013). Some authors have proposed that this layer may contribute as a fomite in spreading fish pathogens to adjacent farms (e.g. Stene et al., 2013). Because the ocean current, in general, has a higher speed at the surface (e.g. Garrison, 2007), one could therefore imagine that lipid-associated pathogens on the sea surface would have a possibility to travel over longer distances, more or less

unaffected by a dilution effect. The lipid film may offer protection and counteract a natural dilution effect, and therefore contribute to disseminate a potential high viral load to adjacent farms. Consistent with this hypothesis, results in the current study indicate that the lipid-associated virions in this layer may be partially protected from UV radiation, as infective virions were re-isolated after 3 hours in the beakers exposed to UVR containing oil, as opposed to beakers containing no oil, where no isolation was possible after the start of the experiment. Furthermore, as the inoculum were mixed with the oil before it was added to the water in the beakers, the virions were likely to be lipid-associated rather than distributed in the water. Consequently, lipid-associated virions in beakers containing oil were exposed to higher UV irradiance than virions in the other beakers, given that the oil has a positive buoyance, and hence, were closer to the UV lamp. However, as the majority of virions were concentrated in 115 mL of oil, as opposed to the other beakers where the virions were distributed in 4 L of water, the concentration of virions could be an effect itself, given that a minimum of virions is required to be an infective dose. Moreover, SAV RNA in water samples from beakers containing oil yielded lower Ct-values by real-time RT-PCR than beakers without oil, indicating that more virions were re-isolated at each sampling point from beakers containing oil. Additionally, the amount of oil used in this study formed a layer of approximately 5 mm on the surface, which is most likely thicker than a lipid film on the sea surface. Hence, the potential protection the oil provided in this study is probably more prominent than actual conditions. Absorption of UV radiation in organic molecules, e.g. lipids, depends on presence of chromophores (Anil Kumar and Viswanathan, 2012), and absorbance will vary between different oils, depending on the oil's chemical properties. However, the oil composition used in this study's ability to absorb wavelengths in the UV spectrum, remains unknown. Anil Kumar and Viswanathan (2012) showed that cod liver oil transmit 60-75 % of UV rays in the UV-B spectrum, and around 100 % in the UV-A spectrum. Although UV-B has the most damaging effect on nucleic acids (see. Fig. 2), the UV irradiances reaching the earth's surface on a sunny day in the summer consists of 96.5 % UV-A and 3.5 % UV-B (Korać and Khambholja, 2011), and it is therefore uncertain how much protection cod liver oil would offer. However, whether cod liver oil exhibit the same properties as salmon oil, regarding UV transmission, is not known.

The UV irradiances used in the experiment were supposed to simulate natural irradiances in surface waters during the summer on the western coast of Norway. However, the measured UV index in the study revealed a value of 22 at the surface of the water and a mean value in the beakers of 11, which are approximately four times and twice, respectively, the UV index-value around Bergen, Norway at noon on a sunny day during the summer (<http://www.nrpa.no/uvnett/dagsverdier.aspx>). Thus, it is likely that survival times for SAV exposed to natural sunlight during the summer in surface coastal waters in western Norway are at least 2-4 times longer than experimental survival times for SAV exposed to artificial UVR presented in this study. Albeit, as UV index is a value based on wavelengths in the UV spectrum weighted for ability to induce erythema on human skin (Vanicek et al., 2000), the exact virucidal effect of the same UV irradiances are not known. However, of the wavelengths present in sunlight reaching the earth's surface, those around 300 nm (UV-B), which were included in this study (see Fig. 4), are known to have the most damaging effects on both human skin (Setlow, 1974, McKinlay and Diffey, 1987) and viral nucleic acid (Caldwell, 1971, Setlow, 1974, see Fig. 2.). In the natural environment, the UV index-value will vary during the day and reach 0 when the sun is down, whereas in this study, UV index was constant during the 72 hours the experiment lasted. Moreover, during the rest of the year, and especially in areas where UV irradiance is weaker (e.g. in northern counties of Norway where UV index-values are rarely higher than 3-4), SAV survival times are in all likelihood significantly longer than survival times for SAV exposed to UVR in the current experiment. Additionally, during the winter months, SAV particles will survive for longer periods as, according to the findings of Graham et al. (2007), SAV survival is inversely proportional to temperature. Considered that survival times in natural seawater in beaker 1 and 2, not exposed to UVR, were at least 48 and 24 hours, respectively, and survival times in beakers exposed to UVR were less than 6 hours, it is likely that survival times for SAV in the natural environment is somewhere between 6 and 72 hours.

Nevertheless, results from the current study support that UVR has an impact on infectivity before virions and their nucleic acid are destroyed, as infectivity was lost in all beakers exposed to UVR somewhere within the first 6 hours while Ct-values of obtained SAV RNA from water samples yielded a steady decline over the 72 hours the experiment lasted. Thus, presence of viral RNA detected by real-time

RT-PCR is not a reliable indication of infective virions. These results coincide with earlier studies (e.g. Suttle and Chen, 1992, Jacquet and Bratbak, 2003).

Conclusions and future perspectives

Based on the results in this study, it is anticipated that SAV virions will survive for less than 72 hours in the natural environment, given a seawater temperature around 10°C. This, along with previous studies regarding survival times in natural seawater, further support that waterborne, horizontal transmission does occur at an infected farm. However, as results from the current study show, survival of SAV was reduced in natural seawater, and particularly in seawater exposed to ultraviolet radiation, compared to the sterile control. Given that waterborne virions will be diluted in the water column, it is uncertain if an infective dose of SAV virions can be transported by passive drift from an infected farm to other farms. Nevertheless, PD outbreaks have a tendency to cluster in time and space, and the impact of a lipid film on the sea surface as a fomite in transmitting infective virions from one farm to others, remains to be clarified. Results from the current study indicate that a lipid film may enhance SAV survival in the natural environment, or at the very least has a concentrating effect.

Hydrodynamic models can contribute to clarify potential transmission pathways for fish pathogens in aquaculture, but the survival of the respective pathogens in natural environment should be further investigated to determine the actual spreading potential. The possible protection a lipid film may offer to waterborne virions is of particular interest and should be further investigated, as the lipid film also may counteract a natural dilution effect of waterborne virions in the water column.

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