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The interaction of infectious salmon anaemia virus (ISAV) with the blue mussel, *Mytilus edulis*

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Abstract

Integrated multi-trophic aquaculture (IMTA) is an alternative approach to mono-culture aquaculture that reduces environmental impacts of commercial aquaculture systems by combining the cultivation of fed species with extractive species. Shellfish play a critical role in IMTA systems by filter-feeding particulate-bound organic nutrients. They may also increase or decrease disease risk on farms by serving as reservoirs or barriers for important finfish pathogens such as infectious salmon anaemia virus (ISAV). This study aimed to optimize culture and molecular assays in shellfish tissues and to determine the fate of ISAV in mussels, *Mytilus edulis*. To determine detection limits, qRT-PCR and culture assays in both CHSE- and ASK cells were optimized in ISAV-inoculated mussel tissue homogenates. Both qRT-PCR and culture assays performed in ASK cells had comparable detection limits of $10^{2.8}$ $TCID_{50}$ mL^{-1}. The ISAV RNA genome was consistently detected in digestive gland tissue of ISAV-exposed mussels. Viable ISAV was not detected in mussel tissues by culture analysis in CHSE- and ASK cells. The fact that qRT-PCR analysis resulted in positive cycle threshold (CT) values that corresponded to the detectable range of ISAV in ASK culture assays suggests that little to no viable ISAV particles are present in the mussel tissues.

Keywords: infectious salmon anaemia virus, integrated multi-trophic aquaculture, mussels, qRT-PCR

Introduction

Integrated multi-trophic aquaculture (IMTA) is an evolving alternative approach to aquatic mono-culture that reduces potential environmental impacts of commercial aquaculture systems by combining the cultivation of fed aquaculture species (finfish) with extractive aquaculture species (e.g. shellfish and seaweed) (Chopin, Buschmann, Halling, Troell, Kautsky, Neori, Kraemer, Zertuche-Gonzalez, Yarish & Neefus 2001; Neori, Chopin, Troell, Buschmann, Kraemer, Halling, Shpigel & Yarish 2004; Ridler, Wowchuk, Robinson, Barrington & Chopin 2007). The IMTA increases the sustainability and profitability of finfish culture as the organic particulate wastes, such as uneaten fish food, can be removed by the shellfish extractive component and dissolved inorganic nutrients extracted by the seaweed component (Ridler *et al.* 2007; Troell, Joyce, Chopin, Neori, Buschmann & Fang 2009). Shellfish play a critical role in an IMTA system by extracting particles and particulate-bound organic nutrients, however, they may also influence pathogen dynamics. Bivalves are well-known bio-accumulators and may serve as a reservoir or as a sink for important finfish pathogens (Mortensen, Bachere, Gall & Mialhe 1992; Mortensen 1993; Paclibare, Evelyn, Albright & Prosperi-Porta 1994; Skår & Mortensen 2007; Molloy, Peitrak, Bouchard & Bricknell 2011; Pietrak, Molloy, Bouchard, Singer & Bricknell 2012; Wangen, Karlsbakk, Einen, Ottem, Nylund & Mortensen 2012). Growers on the eastern coast of the United States are interested in integrating blue mussel, *Mytilus edulis*, cultivation with Atlantic salmon (*Salmo salar*) cultivation but are particularly concerned about the potential for mussels to serve as reservoirs for pathogens such as infectious salmon anaemia virus (ISAV), the aetiological agent of infectious salmon anaemia (ISA).

Infectious salmon anaemia is a viral disease of salmonids, characterized by lethargy, acute anae-

mia, haemorrhagic liver necrosis, ascites, renal tubular necrosis and high mortalities (Evensen, Thorud & Olsen 1991; Rimstad & Mjaaland 2002). The disease initially appeared in the Norwegian Atlantic salmon industry in 1984 (Rimstad APMIS review 2002) and later appeared in Scotland, Canada, the U.S., the Faroe Islands and Chile (Mullins, Groman & Wadowska 1998; Rodger, Turnbull, Muir, Millar & Richards 1998; Bouchard, Keleher, Opitz, Blake, Edwards & Nicholson 1999; Devold, Krossøy, Aspehaug & Nylund 2000; Kibenge, Lyaku, Rainnie & Hammell 2000; Bouchard, Brockway, Giray, Keleher & Merril 2001; Rimstad & Mjaaland 2002).

Infectious salmon anaemia virus is an enveloped virus of the *Orthomyxoviridae* family with a particle size of 80–120 nm (Falk, Namork, Rimstad, Mjaaland & Dannevig 1997; Bouchard *et al.* 1999; Krossøy, Hordvik, Nilsen, Nylund & Endresen 1999; Sandvik, Rimstad & Mjaaland 2000). Like the influenza virus, also an *Orthomyxovirus*, the ISAV genome consists of eight segments of negative sense single-stranded RNA (Mjaaland, Rimstad, Falk & Dannevig 1997). The ISAV particles appear to be relatively unstable in the environment compared with other viruses such as infectious pancreatic necrosis virus, a non-enveloped *Birnavirus*. The ISAV particles are inactivated below a pH of 5.7 with an upper limit of a pH of 9. Viral replication is reduced at temperatures above the optimal temperature of 15°C (Falk *et al.* 1997). Furthermore, prolonged exposure of ISAV to 4°C seawater results in reduction of titre (Rimstad & Mjaaland 2002). Due to the physiology of ISAV in the environment, it is possible that mussels would inactivate ISAV particles.

There is evidence indicating that bivalves may act as either bio-filters or reservoirs for finfish pathogens (Mortensen *et al.* 1992; Mortensen 1993; Paclibare *et al.* 1994; Skår & Mortensen 2007; Molloy *et al.* 2011; Pietrak *et al.* 2012; Wangen *et al.* 2012). In investigating the potential of wild mussel populations acting as potential reservoirs for ISAV, Skår and Mortensen, investigated the ability of mussels to accumulate ISAV in tissues (Skår & Mortensen 2007). Skår and Mortensen did use molecular assays to detect the presence or absence of ISAV in mussel tissues, however, they did not determine the quantity of virus in ISAV-exposed mussels by molecular- or culture-based methods. Isolating ISAV from tissues can be difficult and results can vary depending on the fish cell line used (Rolland, Bouchard, Coll & Winton 2005). Furthermore, isolation of virus from mussel tissue can be difficult due to the severe cell cytotoxicity caused by mussel digestive gland (hepatopancreas) homogenates. To date, there is no published protocol for isolation of fish viruses, in particular ISAV, from shellfish tissues. Culture assays in ASK- and CHSE cells were developed for the isolation of ISAV from mussel tissue homogenates using a low-speed centrifugation technique that improves ISAV infectivity (Molloy, Thomas, Hoyt & Bouchard 2012). To obtain a more complete understanding of the fate of ISAV in the blue mussel, quantitative RT-PCR (qRT-PCR) analysis and quantitative culture analysis of tissue samples from ISAV-exposed mussels were carried out. To better interpret the data from the mussel exposure trials, qRT-PCR and culture assays in both Chinook salmon embryo (CHSE) -214 cells and in Atlantic salmon kidney (ASK) cells were optimized in ISAV-inoculated mussel digestive gland homogenates.

Materials and methods

Cell culture maintenance and virus propagation

The CHSE-214 cells were maintained in minimum essential medium (MEM) (Invitrogen, Carlsbad, CA) with Earle's salts supplemented with 10% foetal bovine serum (FBS) (Invitrogen) at 15°C. The ASK cells were maintained in Leibovitz-15 (L-15) cell culture medium (Invitrogen) with 10% FBS at 15°C.

The Charlie Cove Back Bay (CCBB) New Brunswick ISAV isolate was obtained from Kennebec River Biosciences, Richmond, ME. Virus used in mussel exposure trial 1 was propagated in CHSE-214 cells grown at 15°C in MEM containing 5% FBS. The ISAV used in the detection limit experiment in ISAV-inoculated mussel digestive gland tissues and in mussel exposure trial 2 was propagated in ASK cells grown at 15°C in L-15 cell culture medium with 5% FBS. When cells demonstrated 75% CPE, the virus was harvested and stored at −80°C. Prior to any experiments, the viral suspension was filtered through a 0.45-μm filter to remove any cell aggregations.

Culture analysis of mussel digestive glands and water samples

The CHSE-214 cells or ASK cells were transferred to 24-well or 96-well culture plates. Cells were

allowed to attach and acclimate for 24 h at 15°C to become 75–80% confluent. To prepare tissue samples for culture analysis, digestive gland (hepatopancreas) tissues were weighed, diluted 1:5 in sterile PBS and gently homogenated. Tissue homogenates were diluted tenfold in MEM with Earle's salts or L-15 media containing gentamicin (50 μg mL^{-1}) before filtering through 0.45-μm filters. Water samples were prepared for culture analysis by filtering samples through 0.45-μm filters.

To quantify ISAV in mussel digestive gland tissues and in water samples, an end point dilution assay, the 50% tissue culture infectious dose ($TCID_{50}$) was determined in CHSE-214 cells in trial 1 and in CHSE cells and ASK cells in trial 2. Serial tenfold dilutions in MEM with Earle's salts (CHSE cells) or L-15 media (ASK cells) containing gentamicin (50 μg mL^{-1}) were carried out on all filtrates, except those of negative controls. To quantify virus, each dilution was added in 100-μL volumes to four wells of a 96-well plate containing CHSE-214 cells or ASK cells. Filtrates from negative control tissues or water samples were tested for presence or absence of ISAV by inoculating 100-μL volumes into wells of 24-well plates seeded with CHSE-214 cells or ASK cells after removal of media. All plates were centrifuged for 30 min at 15°C at 500 *g*, to enhance viral adsorption (Epsy, Smith, Harmon & Kendall 1986; Molloy *et al.* 2012). To prevent cell cytotoxicity, following the viral adsorption, inoculum was removed from wells receiving digestive gland homogenate 10^{-1} filtrate dilutions and from wells receiving negative control samples before addition of 1.0 mL of the appropriate fresh medium containing 5% FBS and gentamicin. Plates containing CHSE-214 cells were incubated at 15°C with 5% CO_2 and observed daily for visible CPE for 21 days. Plates containing ASK cells were incubated at 15°C without CO_2. The $TCID_{50}$ was calculated using the method of Reed and Muench (Dougherty 1964).

Mussel maintenance

Market-sized mussels were obtained from commercial mussel growers and maintained in static systems at 10°C in artificial seawater (ASW) (Crystal Seas, Blatimore, MD). Mussels were fed a diet of mixed species algal paste (Innovative Aquaculture, Skerry Bay, BC). Mussels were maintained in static systems containing 0.5 L of ASW per mussel at 10°C in both trials.

The interaction of infectious salmon anaemia virus mussel exposures

Two trials were carried out to determine the fate of ISAV in mussels. Five per cent of mussels were screened by culture and molecular analysis for the presence of ISAV in each of the trials.

In trial 1, 60 mussels were randomly assigned to each of four tanks. The ISAV stock was added to triplicate tanks containing mussels. An equivalent amount of MEM was added to a control tank containing mussels and to a tank containing water only. A sixth system, containing water only, received the ISAV inoculum. The water in each tank was mixed thoroughly and water samples were taken from each of the six tank systems for culture analysis. Water and random triplicate mussel samples were taken at 2-, 24-, 48-, 96- and 144 h post inoculation (hpi). The shell of each mussel was surface disinfected with a 5% sodium hypochlorite solution followed by a swabbing with 70% ethanol. The shell length of each mussel was recorded and digestive gland (hepatopancreas) tissue was removed for culture and molecular analyses. Digestive gland tissues for molecular analysis were stored in RNA later (Ambion, Austin, Tx) for 24 h at 4°C before removing the RNA later and storing at −80°C. Water samples were processed for culture analysis only. Culture analysis of mussel and water samples was carried out in CHSE-214 cells.

Trial 2 was carried out in the same manner as trial 1 with the following modifications. Mussels were randomly assigned to each of four systems until each system contained six mussels. Trial 2 included the same control tanks as in trial 1. After ISAV inoculation, water and random triplicate mussel samples were taken at 24- and 48 hpi and processed for culture and molecular analyses. Culture analyses were carried out on both CHSE-214 cells and ASK cells.

The titre of the stock ISAV was determined by $TCID_{50}$ end point analysis in CHSE-214 cells in trial 1 and in CHSE-214 and in ASK cells in trial 2. In trial 1, the ISAV titre of the water immediately after inoculation was below the detection limit of the $TCID_{50}$ assay performed in CHSE-214 cells. Therefore, the titre was calculated from the titre of the inoculums and assumed to be log 3.7 $TCID_{50}$ mL^{-1}. In trial 2, $TCID_{50}$ endpoint assays were performed on water samples immediately after inoculation in both CHSE-214 and ASK cells.

While ISAV was detected in the time 0 water samples in CHSE-214 cells, the starting ISAV titre in the water could only be calculated from the assay performed in ASK cells (log 3.7 ± 0.1 SE $TCID_{50}$ mL^{-1}).

RNA isolations and quantitative RT-PCR (qRT-PCR)

Each mussel homogenate sample was placed in a 2.0 mL tube containing 600 μL of RLT buffer (Qiagen, Valencia, CA) and a 5 mm stainless steel bead (Qiagen). Samples were further processed in the TissueLyser (Qiagen) twice for 2 min at a frequency of 28 s^{-1}. The RNA extractions were carried out using the RNeasy mini kit (Qiagen) with Qiashredder (Qiagen) and DNase treatment (Qiagen) on the column in the Qiacube automated workstation (Qiagen). The yield and quality of the RNA was assessed using the RNA 6000 Nano LabChip kit (Agilent Technologies, Santa Clara, CA) and the Agilent 2100 Bioanalyzer (Agilent Technologies).

The cDNA was synthesized from 2.0 μg of RNA in 20 μL reactions containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 10 mM DTT, 3 mM $MgCl_2$, 4 mM (each) dNTPs (Applied Biosystems, Carlsbad, CA), random hexamer primers (5 mM) (Applied Biosystems), 20 U of recombinant RNAse inhibitor (Applied Biosystems) and 50 U of Multiscribe reverse transcriptase (Applied Biosystems). Reactions were incubated at 25°C for 10 min, 37°C for 2 h and finally were heat inactivated at 85°C for 5 s.

The qPCR assays were performed using the MX4000 Multiplex Quantitative PCR system (Stratagene, Santa Clara, CA). Reactions were carried out in 25-μL volumes. Primer and probe sequences and methodology reported by Snow, Mckay, Mcbeath, Black, Doig, Kerr, Cunningham, Nylund and Devold (2006) were used to quantify ISAV segment 8 with the exception that an alternate housekeeping gene was used to assess the starting amount of RNA and to normalize the gene-specific product data (Table 1) (Snow *et al.* 2006). Using Primer3 software, a primer/probe set was designed to amplify an 80-bp sequence that straddles an intron in the elongation factor 1α (ELF-1α) gene (Accession No. EU684205; Table 1) (Rozen & Skaletsky 2000). The ELF-1α is consistently expressed throughout the *M. edulis*, gill, mantle, intestine and digestive gland tissues in the presence and absence of viral infection (data not shown). Samples were considered positive if all three qPCR replicate reactions generated threshold cycle (CT) values of ≤ 39. Each sample was analysed by qPCR in triplicate and three samples were analysed per tank per time point. The change in the abundance of ISAV segment 8 was normalized to ELF-α RNA and calculated using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001). Positive and no template controls in each of the processes, RNA extraction, cDNA synthesis and qPCR, were carried out through qPCR analysis.

Table 1 Primers and probes used for qRT-PCR analysis

Gene	Primer/probe	Sequence 5'-3'
ISAV segment 8	Forward	CTACACAGCAGGATGCAGATGT
ISAV segment 8	Reverse	CAGGATCCGGAAGTCGAT
ISAV segment 8	Taqman Probe	FAM-CATCGTCGCTGCAGTTC-MGB
elf-1a	Forward	CGGAGTCAACAAGATGGACA
elf-1a	Reverse	AACTGCTGACTTCCTTCTGGA
elf-1a	Taqman Probe	FAM-CAGTGAAGCCCGATTCAT GGA-MGB

To validate the ISAV qRT-PCR assay using the mussel ELF-1α gene as a housekeeping gene, the relative efficiencies of ISAV segment 8 and ELF-1α primer/probe sets were compared. The cDNA was synthesized from RNA isolated from mussel digestive gland homogenates after inoculation with stock ISAV. Triplicate qPCR reactions targeting both ISAV segment 8 and mussel ELF-1α were performed on serial tenfold dilutions of the cDNA. The ΔCT ($CT_{ISAV} - CT_{ELF\text{-}1\alpha}$) was plotted against the log RNA input to create a semi-log regression line. The slope of the line was less than 0.1 ($m = 0.011$) indicating that the amplification efficiencies of the ISAV segment 8 primer/probe set and the ELF-1α primer/probe set are approximately equal. For each triplicate set of qPCR reactions targeting ISAV segment 8 and mussel ELF-1α, cycle number plotted against dilution factor resulted in a linear plots with a slope of −3.32, which indicates efficient amplification in both assays.

Detection limit of $TCID_{50}$ end point assay and qPCR assay in ISAV-inoculated mussel digestive gland homogenates

Digestive glands from five mussels were harvested, pooled, weighed and diluted twofold in sterile PBS.

A uniform homogenate of the digestive gland tissue was divided equally into 8 × 900-μL samples. Serial tenfold dilutions of stock ISAV, ranging in titre from10^6 to 1.0 $TCID_{50}$ mL^{-1} were performed in MEM cell culture media. Each virus dilution was added in 100-μL volumes to seven of the eight homogenate samples and thoroughly mixed to achieve predicted titres ranging from 10^5 to 0.1 $TCID_{50}$. The MEM was added to the eighth homogenate sample, which served as a negative control for the $TCID_{50}$ and qPCR assays. The RNA was isolated from duplicate 75-mg samples taken from each of the eight homogenates. Remaining homogenates were processed for $TCID_{50}$ analysis in both CHSE-214 and ASK cells. For $TCID_{50}$ assays performed in CHSE-214 cells, digestive gland homogenate samples were diluted 1:9 (wt vol^{-1}) in MEM containing gentamicin (50 μg mL^{-1}) (MEM-G) and filtered through 0.45-μm filters. For $TCID_{50}$ assays performed in ASK cells, digestive gland homogenate samples were diluted 1:9 in L-15 media containing gentamicin (50 μg mL^{-1}) (L-15G) and filtered on 0.45-μm filters. A 2.5-fold dilution was carried out before preparing serial tenfold dilutions to 10^{-10} in unsupplemented MEMG or L-15G media. The $TCID_{50}$ assays were carried out as described above.

Statistics

For qRT-PCR data, a one-way analysis of variance (ANOVA) was performed on ΔΔCT values with alpha set at 0.05. Studentized residuals were tested for normality and equal variance within treatments using the Shapiro-Wilks test (α=0.05) and Levene's test for equal variance (α=0.05) respectively.

Results

ISAV mussel exposure trial 1

To determine if mussels remove ISAV from the water column and accumulate viable ISAV in their tissues, mussels were exposed to ISAV at a known titre for up to 144 h. The ISAV was not detected by culture or molecular analyses in any of the control mussel samples. The ISAV was also not detected by culture analysis in CHSE-214 cells in any of the mussel digestive gland samples taken at any time point. However, ISAV segment 8 RNA was detected by qRT-PCR in nearly all the mussel samples from ISAV-inoculated tanks (Fig. 1). There was no significant difference in the relative abundance of ISAV segment 8 RNA in mussels harvested at the various time points ($F = 2.26$; $P = 0.1353$).

ISAV detection limit of qRT-PCR and culture analyses in mussel digestive gland tissues

The ISAV detection limits in mussel digestive gland tissues were determined for both the qRT-PCR assay and the $TCID_{50}$ assay performed in CHSE-214 cells and in ASK cells, a cell line known to be highly susceptible to ISAV infection. Mussel digestive gland homogenates were inoculated with serial tenfold dilutions of ISAV stock. The titre of the ISAV stock, $10^{5.8}$ and $10^{7.5}$ $TCID_{50}$ mL^{-1}, was determined by $TCID_{50}$ endpoint analysis in CHSE-214 cells and ASK cells respectively. Using the titre determined in CHSE-214 cells, the predicted titres of the mussel digestive gland homogenates ranged from $10^{4.8}$ to 10^{-1} $TCID_{50}$ mL^{-1}. Using the stock titre determined in the ASK cells, the predicted titres of the mussel digestive gland homogenates ranged from $10^{6.5}$ to $10^{0.5}$ $TCID_{50}$ mL^{-1}. To simplify comparisons, predicted ISAV titres will refer to those calculated from the

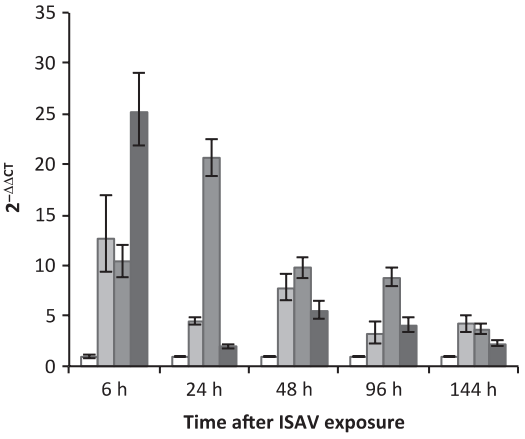

Figure 1 The infectious salmon anaemia virus (ISAV) segment 8 relative abundance in mussel digestive glands at 6-, 24-, 48-, 96- and 144 h after exposure to MEM (white bar) or after exposure to ISAV (shaded bars represent triplicate tanks) as measured with Taqman quantitative RT-PCR in trial 1. Graphs represent average values ± standard error of the mean with $n = 3$.

original stock titre determined in CHSE-214 cells, unless otherwise specified.

The qRT-PCR detected ISAV RNA in mussel homogenates with predicted titres ranging from $10^{4.8}$ to $10^{1.8}$ $TCID_{50}$ mL^{-1} with a linear increase in CT value as the predicted log ISAV titre decreased ($R^2 = 0.99$) (Fig. 2). While ISAV RNA was detected in the homogenate samples with a predicted ISAV titre of $10^{1.8}$ $TCID_{50}$ mL^{-1}, the CT values were not consistently less than 40. Therefore, the ISAV titre of $10^{2.8}$ $TCID_{50}$ mL^{-1} homogenate was considered a more reliable detection limit.

Viable ISAV was detected in mussel homogenates with predicted ISAV titres ranging from $10^{3.8}$ to $10^{4.8}$ $TCID_{50}$ mL^{-1} by culture analysis performed in CHSE-214 cells (Fig. 2). The sensitivity of $TCID_{50}$ assay performed in the ASK cells was higher than that performed in CHSE-214 cells by one order of magnitude (Fig. 2). Culture analysis performed in ASK cells detected viable ISAV in homogenates with predicted titres as low as $10^{2.8}$. The detection limit of the $TCID_{50}$ assay performed in ASK cells corresponded with a CT value of 37.08 (Fig. 2). The ISAV titre determined by endpoint analysis in CHSE-214 cells was lower than that of the predicted ISAV titre calculated from the stock titre determined in CHSE-214 cells. Similarly, the ISAV titre determined by endpoint analysis in the ASK cells was lower than the predicted ISAV titre calculated from the stock titre determined in ASK cells.

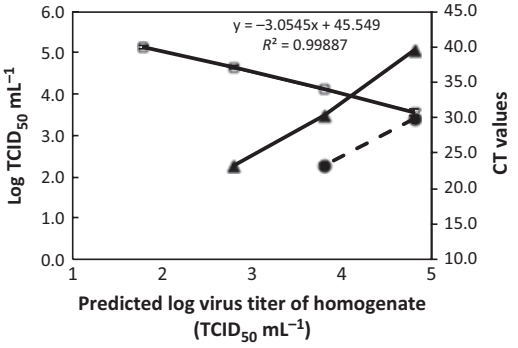

Figure 2 Log tissue culture infectious dose ($TCID_{50}$) infectious salmon anaemia virus (ISAV)-inoculated mussel digestive gland homogenates determined in Atlantic salmon kidney (ASK) cells (triangles) or Chinook salmon embryo (CHSE)-214 cells (circles) and average cycle threshold (CT) values (open squares) as measured with Taqman quantitative RT-PCR using primers specific for ISAV segment 8. CT values represent average values ± standard error of the mean with $n = 2$.

ISAV mussel exposure trial 2

We were unable to determine if the negative culture results in trial 1 were due to inactivation of ISAV particles by the mussel or due to ISAV titres below the detection limit of the $TCID_{50}$ assay performed in CHSE-214 cells. Therefore, we repeated the ISAV mussel exposure trial and analysed water and mussel tissue samples at 24- and 48 hpi using culture-based assays in both CHSE-214 cells and in the more sensitive ASK cells. Mussel tissue samples were also analysed using molecular assays.

In trial 2, $TCID_{50}$ endpoint assays were performed in both CHSE-214 and ASK cells. While ISAV was detected in the time 0 water samples in CHSE-214 cells, the starting ISAV titre in the water could only be calculated from the assay performed in ASK cells. The average starting ISAV titre in ISAV-inoculated tanks containing mussels was log 3.7 ± 0.1 SE ($n = 3$) $TCID_{50}$ mL^{-1} of water (Fig. 3). The starting ISAV titre in an ISAV-inoculated control tank, containing water only, was log 4.0 $TCID_{50}$ mL^{-1} of water (Fig. 3). At 48 hpi there was a slight decrease in the ISAV titre of the water to log 3.2 $TCID_{50}$ mL^{-1} of water in the control tank without mussels (Fig. 3). In ISAV-inoculated tanks containing mussels, there

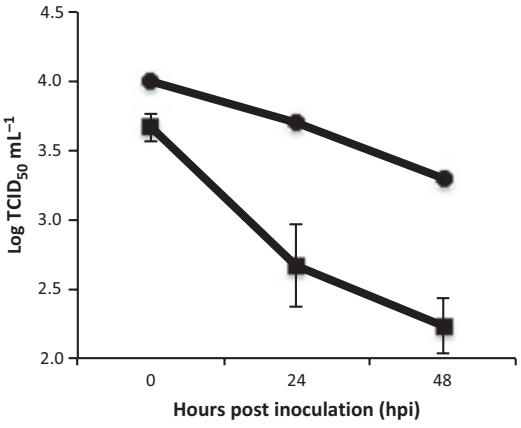

Figure 3 The infectious salmon anaemia virus (ISAV) titre (log $TCID_{50}$ mL^{-1}) of water over time in ISAV-inoculated tanks containing mussels (squares) or lacking mussels (circles). Graphs represent average values ± standard error of the mean with $n = 3$.

was a decrease in ISAV titre of the water by 1.5 orders of magnitude. Viable ISAV was not detected in control mussels or in ISAV-exposed mussels by culture analysis using CHSE-214 cells or ASK cells.

Infectious salmon anaemia virus segment 8 RNA was detected by qRT-PCR in mussel digestive gland tissue from ISAV-exposed mussels at both 24- and 48 hpi (Fig. 4). There was no significant difference in ISAV segment 8 RNA abundance at 24- and 48 hpi ($F = 4.66$; $P = 0.1$). The abundance of ISAV segment 8 RNA appears to be comparable to ISAV RNA abundance at the same time points in trial 1. The average CT values for ISAV-positive mussels at 24- and 48 hpi were 37.9 ± 0.6 SE ($n = 9$) and 38.7 ± 0.4 SE ($n = 9$) respectively. The CT values for ISAV-positive mussels were as low as 35.9 at 24 h (Table 2). All control mussels were negative for ISAV by qRT-PCR.

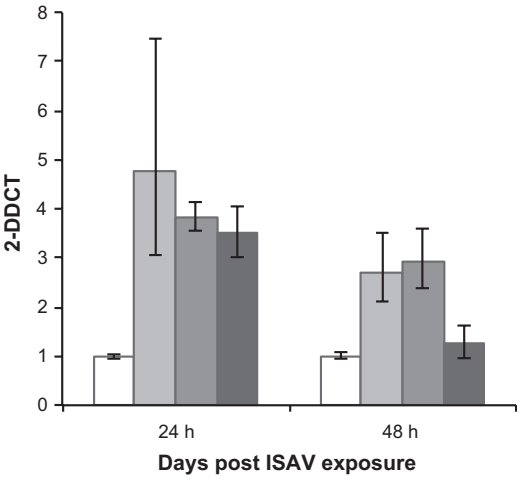

Figure 4 Average infectious salmon anaemia virus (ISAV) segment 8 relative abundance in mussel digestive glands at 24- and 48 h after exposure to minimum essential medium (MEM) (white bar) or after exposure to ISAV (shaded bars represent triplicate tanks) as measured with Taqman quantitative RT-PCR in trial 2; Graphs represent average values ± standard error of the mean with $n = 3$.

Discussion

Integrated multi-trophic aquaculture is an attractive alternative to mono-culture aquaculture because of its potential reduction in environmental impacts of commercial aquaculture systems while increasing the sustainability and profitability of finfish culture. Shellfish components in an IMTA system play a critical role by extracting particulate bound organic nutrients, such as uneaten fish food (Troell, Halling, Noeri, Chopin, Buschmann, Kautsky & Yarish 2003), however, they may also influence pathogen dynamics. Bivalves are well-known bio-accumulators and may serve as a reservoir or as a sink for important finfish pathogens. In particular, salmon growers on the East coast of North America have voiced concern about the potential for mussels to serve as reservoirs for ISAV. To better understand the fate of ISAV in mussels, we need to determine the viral load in mussels post exposure to ISAV.

Table 2 The average cycle threshold (CT) value resulting from qRT-PCR analysis of digestive gland tissue harvested from nine replicate mussels after exposure to infectious salmon anaemia virus (ISAV) in trial 2. Values represent the average CT value generated from triplicate qPCR reactions ± the standard error of the mean

	Hours of exposure	
Replicate	24 h	48 h
1	35.9 ± 0.3	39.7 ± 0.3
2	38.6 ± 0.7	36.9 ± 0.5
3	38.1 ± 1.0	39.6 ± 0.4
4	No CT	39.6 ± 0.4
5	36.1 ± 0.2	37.4 ± 0.5
6	39.8 ± 0.2	37.2 ± 0.6
7	36.0 ± 0.0	38.5 ± 0.9
8	39.6 ± 0.4	39.7 ± 0.3
9	37.3 ± 0.5	39.5 ± 0.5

We have developed culture assays in two fish cell lines for the detection of ISAV in mussel tissue homogenates. These culture assays in conjunction with quantitative RT-PCR assays allowed us to determine whether or not mussels bio-accumulate or inactivate ISAV after exposure to this virus. Clearly mussels are capable of filtering ISAV particles from the water column. The ISAV RNA was detected in mussel digestive gland tissues (Fig. 1). There appears to be a decrease in the relative abundance of ISAV segment 8 RNA in mussel tissues over time; however, the difference in segment 8 abundance was not significant. This may be due to the large variation in abundance between replicate tanks, for example at 24 hpi (Fig. 1). The presence of ISAV RNA indicated the presence of ISAV particles in mussel digestive gland tissues, however, we were unable to determine if these particles were viable. All mussel digestive gland

samples were negative for ISAV by $TCID_{50}$ analysis in CHSE-214 cells. It is possible that viable ISAV particles were present in the mussel tissues, however, at a titre that was below the detection limit of the $TCID_{50}$ assay. Alternatively, mussels may have inactivated the ISAV particles and therefore ISAV was detectable only by molecular-based methods.

To further interpret whether viable ISAV particles were present in mussel tissues after ISAV exposure, we compared the detection limits of the qRT-PCR assay and the culture-based assay in ISAV-inoculated mussel digestive gland homogenates. The CT values generated from qRT-PCR analysis were compared with the $TCID_{50}$ mL^{-1} values determined in CHSE cells and in ASK cells, a cell line more sensitive to ISAV infection (Rolland, Bouchard & Winton 2003) (Fig. 2). The qRT-PCR assay detected ISAV RNA in mussel digestive gland homogenates with predicted ISAV titres as low as $10^{1.8}$ $TCID_{50}$ mL^{-1}, although triplicate qPCR reactions did not consistently generate CT values less than 40. Therefore, the reliable detection limit of this qRT-PCR assay in mussel digestive gland tissues should be considered $10^{2.8}$ $TCID_{50}$ mL^{-1} of mussel digestive gland homogenate. The qRT-PCR assay detection limit is thus comparable to the detection limit of the $TCID_{50}$ assay performed in the ASK cells (Fig. 2).

The $TCID_{50}$ assay performed in the ASK cells was more sensitive than that performed in CHSE cells by 1 order of magnitude (Fig. 2). The ASK cells are well known to be more sensitive than CHSE cells in detection of ISAV (Rolland *et al.* 2005). We included CHSE cells in our experiments because they are as susceptible to ISAV infection as ASK cells if treated with centrifugation after inoculation with diluted ISAV stock (Molloy *et al.* 2012) and because their fast growth rate allows analysis of high numbers of samples. However, ASK cells are more sensitive than CHSE cells in detecting ISAV in tissue homogenates of mussel digestive gland but also of Atlantic salmon kidney. In a similar detection limit experiment carried out on ISAV-inoculated Atlantic salmon kidney homogenates, qRT-PCR and $TCID_{50}$ assays performed in ASK cells were more sensitive than the $TCID_{50}$ assay performed in CHSE cells (Molloy *et al.* 2012). Interestingly, the $TCID_{50}$ assay performed in ASK cells was more sensitive than the qRT-PCR assay when analysing Atlantic salmon kidney tissues.

In both $TCID_{50}$ assays performed in CHSE cells and ASK cells, the measured $TCID_{50}$ mL^{-1} of mussel digestive gland homogenate was lower than the predicted $TCID_{50}$ mL^{-1} in each of the respective cell lines. The lower measured titres may be due to inactivation of ISAV particles by enzymes, such as lipases or proteases, present in the digestive gland tissues.

A second ISAV mussel exposure trial was carried out to determine if viable ISAV could be detected in ISAV-exposed mussels using the more sensitive ASK $TCID_{50}$ assay. Due to the higher sensitivity of the ASK cells, it was possible to monitor ISAV titres in the water of ISAV-inoculated tanks. This clearly demonstrated that viable ISAV was available to mussels for filtration. Furthermore, the ISAV titre in the water appeared to be dramatically lower at 48 hpi in tanks containing mussels when compared with the treatment lacking mussels (Fig. 3).

The fact that we were unable to detect viable ISAV in any of the mussel samples that were clearly positive by qRT-PCR provides implied evidence that mussels are inactivating the ISAV particles. The ISAV detection limits in mussel homogenates for the qRT-PCR assay and the culture assay in ASK cells were the same (Fig. 2). A CT value of 37 corresponded to the ISAV detection limit for the culture assay in ASK cells. Therefore, the culture assay in ASK cells should be able to detect ISAV in samples with corresponding CT values of 37 or less if the ISAV particles are viable. The ISAV RNA was detected in nearly all of the ISAV-exposed mussels at 24- and 48 hpi at relative abundances comparable to the 24- and 48 h time points in trial 1 (Figs. 1 and 4). Furthermore, the CT values generated by qRT-PCR analysis of multiple mussel digestive gland samples in trial 2 were less than or equal to 37 and yet no viable ISAV was detected by culture analysis in ASK cells (Table 2). While it is possible we were unable to detect viable ISAV particles via culture because the mussels took up non-viable ISAV particles only from the water column, this is highly unlikely as viable ISAV was detected at all three time points in trial 2 (Fig. 3).

We are not completely able to rule out the possibility that there is still viable ISAV present in the tissues at levels below the detection limit of the culture assay. However, this work, together with the earlier study by Skår and Mortensen indicates that ISAV is quickly removed from the water

column by blue mussels and inactivated (Skår & Mortensen 2007). Skår and Mortensen used molecular methods to demonstrate the ability of mussels to uptake ISAV particles and eliminate ISAV from their tissues after 4 days of depuration. They did not, however, determine viral load by culture or qRT-PCR methods. They elegantly demonstrated that ISAV-loaded mussels after 24 h of depuration likely contain little to no viable ISAV particles by injecting homogenates from these mussels intraperitoneally into ISAV-naïve Atlantic salmon. While no fish developed clinical ISA, one fish out of 25 was weakly positive by qRT-PCR. Intraperitoneal injection of Atlantic salmon with homogenates prepared with ISAV-loaded mussels after 96 h of depuration did not result in development of clinical ISA or ISAV-positive fish indicating elimination of viable ISAV particles from the mussel tissues. It is important to note that neither our study nor the Skår and Mortensen study analysed pseudofaecal pellets released from ISAV-exposed mussels. There is the potential for some filtered ISAV particles to bypass the digestive system and be released back into the environment as viable particles entrapped in pseudofaecal pellets.

This study, in conjunction with the Skår and Mortensen study, clearly demonstrates that, in static systems, mussels are capable of filtering ISAV particles from the water column and likely inactivate those particles. The results from this study indicate that it is highly unlikely that co-cultured mussels on Atlantic salmon farms will act as ISAV reservoirs and increase the risk of ISA outbreaks. The small ISAV particle size, approximately 100 nm in diameter, makes it unlikely that mussels could efficiently filter ISAV from the water column; however, it would be interesting to determine if large masses of filter-feeding mussels, consistent with the quantities of mussels on IMTA farms, could significantly reduce the risk of ISA in Atlantic salmon (Bouchard *et al.* 1999). Finally, this study provided optimized culture and molecular assays for the detection of ISAV in mussel tissues. The culture assay may be adjusted for the detection of other viral pathogens in mussel digestive gland tissues.

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