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Fate of redspotted grouper nervous necrosis virus (RGNNV) in experimentally challenged Manila clam *Ruditapes philippinarum*

E. Volpe[1], N. Pagnini[2], P. Serratore[1], S. Ciulli[1,*]

[1]Department of Veterinary Medical Sciences, University of Bologna, 47042 Cesenatico, FC, Italy

[2]Cesena Campus, University of Bologna, 47042 Cesenatico, FC, Italy

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*Corresponding author: sara.ciulli@unibo.it

ABSTRACT: Redspotted grouper nervous necrosis virus (RGNNV), genus *Betanodavirus*, family *Nodaviridae*, is the causative agent of viral encephalopathy and retinopathy (otherwise known as viral nervous necrosis), and can infect several fish species worldwide. Betanodaviruses, including RGNNV, are very resilient in the aquatic environment, and their presence has already been reported in several wild marine species including invertebrates. In order to investigate the interaction between a bivalve mollusc (Manila clam *Ruditapes philippinarum*) and RGNNV, we optimised a culture-based method. The bioaccumulation of the pathogenic RGNNV by *R. philippinarum* and the potential shedding of viable RGNNV from RGNNV-exposed clams were evaluated through a culture-based method. *R. philippinarum* clearly accumulated viable RGNNV in their hepatopancreatic tissue and were able to release viable RGNNV via faecal matter and filtered water into the surrounding environment. The role of clams as bioaccumulators and shedders of viable RGGNV could put susceptible cohabiting cultured fish at risk. RGNNV-contaminated molluscs could behave as reservoirs for this virus and may modify the virus epidemiology.

KEY WORDS: *Betanodavirus* • RGNNV • *Ruditapes philippinarum* • Manila clam • Bioaccumulation • Shedding • Viral encephalopathy and retinopathy • Marine environment

INTRODUCTION

Redspotted grouper nervous necrosis virus (RGNNV), a virus of the genus *Betanodavirus*, family *Nodaviridae*, is responsible for viral encephalopathy and retinopathy (VER), otherwise known as viral nervous necrosis, a disease that can cause nervous signs and mortality in more than 70 fish species worldwide (Doan et al. 2017). Betanodaviruses are small, icosahedral viruses that contain 2 segments of positive-sense single-stranded RNA. RNA1 (3.1 kb) and RNA2 (1.4 kb) encode a RNA-dependent RNA polymerase of 100 kDa and a major coat protein of 42 kDa, respectively (Mori et al. 1992, Guo et al. 2003). Based on a partial nucleotide sequence of the coat protein gene, betanodaviruses are divided into 4 species: *Striped jack nervous necrosis virus* (SJNNV), *Tiger puffer nervous necrosis virus* (TPNNV), *Barfin flounder nervous necrosis virus* (BFNNV) and *Redspotted grouper nervous necrosis virus* (RGNNV) (Thiéry et al. 2011). NNV is frequently isolated during outbreaks of VER in several farmed fish species, including European sea bass *Dicentrarchus labrax* in the Mediterranean Sea (Panzarin et al. 2012, Vendramin et al. 2013). Moreover, NNV has also been detected in numerous wild marine fish species and invertebrates in the Mediterranean Sea, South Korea, China and Japan (Gomez et al. 2004, 2008a, Ciulli et al. 2007, Liu et al. 2015).

Betanodavirus infection is transmitted horizontally, either directly through the introduction of infected fish, or indirectly by contaminated water and equipment, as well as vertically, through reproduction (Munday et al. 2002). Recent studies have demonstrated that a certain population of apparently healthy wild marine fish carry betanodaviruses, and suggested that these wild fish can be a persistent potential source of virus for cultured fish and the breeding environment (Ciulli et al. 2007, Gomez et al. 2008a, Vendramin et al. 2013). Moreover, a recent finding suggests that trash fish/molluscs can be a source of betanodaviruses for cultured fish and that they pose a serious risk for outbreaks of VER in susceptible cultured fish (Gomez et al. 2010). Currently, no successful therapies or commercial vaccines, apart from one in Japan (OIE 2016), are available to enable adequate control of VER, so disease prevention is based mainly on maintaining proper sanitary procedures, screening activities and correct farm management (Costa & Thompson 2016, Doan et al. 2017).

Pathogenic agents may be spread via water masses, wild carriers, or vectors, and it is well documented that restrictions, such as disinfection process, do not fully ensure the prevention of microorganism spreading (Ciulli et al. 2017) and the control of disease spread by these routes (Mortensen 2000, Mortensen et al. 2006). Several factors affect the pathogenic agents shed into the water, such as dilution, inactivation by UV light or other physical and chemical factors, adsorption

onto the surfaces of suspended particles, and uptake in filter-feeding organisms or particle-feeding plankton (Bitton 1975, Noble & Fuhrman 1997, Sakoda et al. 1997, Skår & Mortensen 2007, Sinton et al. 2002, Wilhelm et al. 2003, Evans et al. 2014). Accordingly, aquatic organism interaction, both in the case of natural or artificial environments, such as integrated multi-trophic aquaculture, can greatly affect the epidemiology of fish infectious diseases. In fact, there is evidence indicating that, when placed closely together, bivalves may act either as bio-filters or as reservoirs for finfish pathogens, as a consequence of their ability to bioaccumulate microorganisms (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). However, the outcome of the interaction may differ on the basis of the morphology and physiology of the pathogen, which influences whether the pathogen remains viable in bivalve mollusc tissues and is shed back into the environment, or is inactivated by the molluscs (Skår & Mortensen 2007, Molloy et al. 2013).

Some studies have investigated the role of wild aquatic organisms such as bivalve molluscs in the interaction with fish pathogens. These studies showed that the infectious salmon anaemia virus (ISAV) is inactivated by blue mussels *Mytilus edulis* (Molloy et al. 2014). In contrast, infectious pancreatic necrosis virus (IPNV) can be transmitted from IPNV-exposed mussels to Atlantic salmon *Salmo salar* (Molloy et al. 2013). The presence of NNV in invertebrates and particularly bivalve molluscs has already been reported (Ciulli et al. 2007, Gomez et al. 2008a), including the Manila clam *Ruditapes philippinarum* in the Mediterranean Sea (Ciulli et al. 2007, Panzarin et al. 2012).

The aim of this study was to examine the bioaccumulation of a pathogenic RGNNV by Manila clams and to investigate the potential shedding of viable RGNNV from RGNNV-exposed clams through a culture-based method.

MATERIALS AND METHODS

Clam maintenance and NNV screening

Batches of market-size Manila clams, hereafter referred to simply as clams, were obtained from a commercial clam trader and were reared in an artificial recirculation system (Adriatic Sea International) supplied with natural seawater, collected from the Adriatic Sea and held at 15°C. Batches of clams were acclimated for 24 h in order to start filtration. Trials were conducted in a static system consisting of 5 l plastic tanks supplied with 2 l natural seawater, aerated and held at 15°C.

Prior to all trials, 30 clams from each batch were screened for the presence of NNV-RNA via a reverse transcription (RT)-PCR assay followed by a nested PCR performed according to methods previously described (Nishizawa et al. 1994, Ciulli et al. 2006). The same molecular detection protocol was applied to confirm the NNV-RNA presence in clam hepatopancreas during the first exposure trial and shedding Trial 1. Moreover, NNV molecular detection was performed on randomly chosen samples (cell lysates) collected directly from 96-well plate culture analysis of hepatopancreas, water and faecal matter to confirm the agreement between cytopathic effect (CPE) and NNV presence.

Cell culture maintenance and virus propagation

Striped snakehead fish cells (SSN-1) were maintained in Leibovitz-15 medium (L-15) (Gibco) supplemented with 1% L-glutamine (Gibco), 1% antibiotic–antimycotic solution (Gibco) and 7.5% foetal bovine serum (FBS) (Gibco) at 25°C. For virus isolation assays, SSN-1 cells were harvested, counted and transferred to 96-well culture plates at a density of 7×10^4 cells cm^{-2}. Cells were allowed to attach and acclimate for 24 h at 25°C in order to achieve 80% confluence.

The previously characterised RGNNV isolate It/351/Sb (Ciulli et al. 2006) was propagated in SSN-1 cells grown at 25°C in L-15 medium containing 2% FBS. When the cells demonstrated a 75% CPE, the cells and supernatant were centrifuged at 500 × *g* (10 min), and the supernatant was stored at –80°C until use. The titre of the stock was determined by 50% tissue culture infectious dose ($TCID_{50}$) end point analysis in SSN-1 cells. The $TCID_{50}$ was calculated according to the Spearman-Karber method (Hierholzer & Killington 1996).

Culture analysis of clam hepatopancreas, faecal matter and water samples

RGNNV presence was detected and quantified by performing $TCID_{50}$ analysis in SSN-1 cells in hepatopancreas tissue, faecal matter and water samples. Water samples were centrifuged at 3000 × *g* (5 min) and the supernatant was filtered through 0.20 μm pore size filters and incubated with 1% antibiotic–antimycotic solution (Gibco) at 4°C overnight. Samples were diluted 10-fold in L-15 with 2% FBS (Gibco). If samples reported negative results, a 2-fold dilution of the supernatants was performed and tested.

Hepatopancreas tissue was weighed, diluted 1:9 (wt/vol) with L-15 containing 2% FBS (Gibco) and homogenised before centrifuging at 3000 × *g* (5 min). The supernatant was diluted 10-fold in L-15 with 2% FBS (Gibco). Faecal matter samples were also centrifuged at 3000 × *g* (5 min), the faecal pellets were weighed, diluted 1:9 (wt/vol) with L-15 containing 2% FBS (Gibco) and incubated

with 1% antibiotic–antimycotic solution (Gibco) at 4°C overnight. The supernatant was diluted 10-fold in L-15 with 2% FBS (Gibco).

For viral titration assays, each dilution was added in 100 µl volumes to 5 wells of a 96-well plate containing 24 h old SSN-1 cells. Negative control wells consisting of L-15 with 2% FBS (Gibco) were included for each plate. The inoculum from wells receiving samples were removed after 1 h viral adsorption period at 25°C to prevent cell cytotoxicity before the addition of 100 µl of L-15 fresh medium containing 2% FBS. The plates were incubated at 25°C and observed daily for visible CPE for 7 d. The titre referred to water samples was expressed as $TCID_{50}$ ml^{-1}. For hepatopancreas tissue and faecal matter samples, culture analysis $TCID_{50}$ values were normalised to (g of hepatopancreas tissue or faecal matter)$^{-1}$ and hereafter referred to as $TCID_{50}$ g^{-1}.

Endpoint dilution assay detection limit in RGNNV-inoculated clam hepatopancreas homogenates

Hepatopancreas from 7 NNV-RNA-negative clams were weighed (mean 69.6 ± 23.2 g), diluted 1:9 (wt/vol) with L-15 containing 2% FBS (Gibco) and homogenised before centrifuging at 3000 × *g* (5 min). Serial 10-fold dilutions of stock RGNNV, ranging in titre from 7.5 to 2.5 log $TCID_{50}$ ml^{-1}, were prepared in L-15 cell culture medium. Each virus dilution was added in 100 µl volumes to 6 of the 7 hepatopancreas homogenates and thoroughly mixed to achieve predicted titres ranging from 6.7 to 1.7 log $TCID_{50}$ ml^{-1}. L-15 containing 2% FBS was added to the seventh homogenised sample, which served as a negative control for the $TCID_{50}$ assays. RGNNV-inoculated hepatopancreas homogenates were processed for $TCID_{50}$ analysis in SSN-1 as described above.

RGNNV clam exposure trial

In order to measure RGNNV uptake in clams, 3 independent exposure trials were performed. In each trial, 60 clams were placed in 5 l plastic tanks containing 2 l of seawater held at 15°C. An air-lift pump circulated the water and provided aeration. RGNNV suspension in L-15 cell culture medium was then added such that the final virus concentration in the tanks was 5 log $TCID_{50}$ ml^{-1}. The clams were left for 24 h to bioaccumulate the virus and then removed. Ten ml of water and random triplicate clam samples were collected at 3, 6 and 24 h post-exposure (hpe). Culture analysis of clam hepatopancreas and water samples was carried out in SSN-1 cells as described above. In the first replicate, clam hepatopancreas and water samples were also tested by the molecular method as described above. Hepatopancreas viral loads were expressed as the mean ± standard deviation (SD) of positive samples obtained from the 3 trials. The samples of faecal matter and water were analysed in 2 repeats, and viral loads are presented as the mean of the positive repeats ± SD.

Clam RGNNV shedding trials

The ability of the clams to shed viable RGNNV into the water through faecal matter was evaluated with 2 subsequent trials.

Trial 1

Shedding Trial 1 was carried out in the same manner as the exposure trial with the following modifications. After 24 hpe, the surface of each clam shell was disinfected with a 1% Virkon®S (DuPont) solution, rinsed under running tap water and then transferred to a clean static system supplied with fresh seawater. During depuration, triplicate clam samples were collected at 1, 2, 5, 6 and 7 days post-depuration (dpd) for culture and molecular assays. Furthermore, after 7 dpd, 10 ml of water and a sample of faecal matter were collected for culture assays.

Trial 2

Shedding Trial 2 was carried out in the same manner as Trial 1 with the following modifications. After the transfer, the clams were moved daily to a clean static system supplied with 100% fresh seawater until 7 dpd. Prior to the daily placements, the shell of each clam was surface disinfected with 1% Virkon®S (Dupont) solution, then rinsed under running tap water. Ten ml of water, faecal matter and triplicate clam samples were harvested for culture assays prior to each daily movement.

Hepatopancreas viral loads of shedding trials were reported as the mean of positive samples ± SD. The samples of faecal matter and water were analysed in 2 repeats, and viral loads are shown as the means ± SD of the positive repeats.

Statistics

Data obtained from the detection limit assay were analysed by a simple linear regression analysis (Prism version 6.0 software, GraphPad Software), considering predicted values as a predictor and measured values as dependent variables. Positive data of culture assays, after testing for normality, were analysed by 1-way ANOVA followed by Tukey's tests to determine statistically the differences among virus titres detected in samples (Prism version 6.0 software, GraphPad Software). Throughout, the level for accepted statistical significance was $p < 0.05$.

RESULTS

Clam maintenance and NNV screening

The NNV screening showed that all the batches involved in the trials were negative for NNV-RNA presence. During all trials, no mortality was recorded in batches of clams used. NNV molecular detection performed on randomly chosen samples collected directly from 96-well plate culture analysis of hepatopancreas, water and faecal matter confirmed the agreement between CPE and NNV presence.

Endpoint dilution assay detection limit in RGNNV-inoculated clam hepatopancreas homogenates

The detection limit for viable RGNNV isolation by culture analysis was 1.7 log $TCID_{50}$ ml^{-1}. Viable RGNNV was detected by culture analyses in hepatopancreas homogenates with predicted titres of log 6.7 to 2.7 log $TCID_{50}$ ml^{-1} (Fig. 1). Titres measured in SSN-1 cells decreased in a linear trend as predicted titres decreased. Linear regression analysis showed a significant association between measured and predicted values ($p = 0.001$). In particular, a decrease in predicted values was associated with a decrease in the measured values ($R^2 = 0.96$, $F[1.4] = 91.64$, $y = 1.206x - 1.562$). However, the determined titres were lower than the predicted titres by a mean of 0.5 ± 0.2 log $TCID_{50}$ ml^{-1}. The most dilute sample in which virus was detected had a predicted titre of 2.7 log $TCID_{50}$ ml^{-1}, although the measured titre was 1.7 log $TCID_{50}$ ml^{-1}. For samples at a predicted titre of 1.7 log $TCID_{50}$ ml^{-1} and lower, no virus was detected by culture assays.

RGNNV clam exposure trial

Uptake by clams of viable RGNNV in the hepatopancreas tissues was evident as early as 3 hpe (Fig. 2). No significant difference was observed among mean viable virus titres detected in clam hepatopancreas collected at the same time points during the 3 clam exposure trials (data not shown). Accordingly, results are expressed as the mean ± SD of all positive samples obtained from the 3 trials.

Eight of the 9 replicate clams were positive by virus isolation at 3 hpe, with a mean titre of 4.0 ± 0.2 log $TCID_{50}$ g^{-1} ($n = 8$). At 6 hpe, 6 of the 9 replicate clams were positive by virus isolation with a mean titre of 4.3 ± 0.4 log $TCID_{50}$ g^{-1} ($n = 6$). After 24 hpe, all sampled clams were positive at virus isolation with a mean titre of 4.4 ± 0.5 log $TCID_{50}$ g^{-1} ($n = 9$). During the exposure trials, the amount of viable RGNNV increased from 4.0 ± 0.2 to 4.4 ± 0.5 log $TCID_{50}$ g^{-1} with no statistical significance (Fig. 2). Moreover, the RGNNV loads measured at different time points in water

samples showed no statistical significance; nevertheless, virus titres decreased from 3.5 ± 0.3 to 2.8 ± 0.2 log$TCID_{50}$ ml^{-1} (Fig. 2). Molecular detection indicated the presence of NNV RNA in clam hepatopancreas and water samples at each time point (data not shown).

Clam RGNNV shedding trials

Trial 1

Viable RGNNV was isolated from all the clams sampled (Fig. 3). The RGNNV mean titre was 5.0 ± 0.2 log $TCID_{50}$ g^{-1}; we found no statistical significance between viable RGNNV amounts at different time points in hepatopancreas samples. After 7 dpd, RGNNV-exposed clams released viable RGNNV into water and through faecal matter (Fig. 3). The titres of viable RGNNV detected in faecal matter and water were 3.5 log $TCID_{50}$ g^{-1} and 1.5 log $TCID_{50}$ ml^{-1}, respectively; these values were statistically lower ($p < 0.05$) than viable RGNNV found in hepatopancreas tissues (5 ± 0.2 log $TCID_{50}$ g^{-1}). Molecular detection confirmed the presence of NNV RNA in clam hepatopancreas samples until 7 dpd (data not shown).

Trial 2

Viable RGNNV was isolated from all hepatopancreas tissues analysed, with a mean titre of 5.1 ± 0.2 log $TCID_{50}$ g^{-1}. RGNNV titre in hepatopancreas at 1 dpd was statistically higher than the titres at 2, 4, 5 and 6 dpd ($p < 0.05$; Fig. 4A). Viable RGNNV was also isolated from water samples at 1, 2, 3 and 4 dpd with a mean titre of 1.3 ± 0.3 log $TCID_{50}$ ml^{-1}. At 2 dpd, only 1 repeat of the water sample reported viable RGNNV. No significant differences were revealed among virus titres detected at different time points (Fig. 4B). In faecal matter, viable RGNNV was isolated from both repeats of all samples with a mean titre of 3.9 ± 0.5 log $TCID_{50}$ g^{-1} except from 1 repeat of the 7 dpd sample. The titre values in faecal matter samples showed variable amounts of viable RGNNV during the trial; in particular RGNNV titration at 1 dpd was statistically higher than the titres at 2 and 6 dpd ($p < 0.05$). No significant differences were evident among other time points (Fig. 4C). In water samples, the titrations showed statistically lower values than in the hepatopancreas tissues and in the faecal matter samples at all tested time points ($p < 0.05$).

DISCUSSION

Bivalve molluscs are well known bioaccumulators and may serve as reservoirs or as natural barriers for important finfish pathogens (Molloy et al. 2013, 2014). Previous studies aimed at investigating virus persistence in bivalve molluscs used both cell culture and molecular methods to evaluate the

viral load in bivalve tissue (Skår & Mortensen 2007, Molloy et al. 2013, 2014). However, due to the presence of PCR inhibitors in bivalve tissues and the inability of molecular methods to distinguish viable from nonviable virus, the most sensitive techniques to evaluate the viral load in bivalve tissues is virus isolation on cell culture (Molloy et al. 2013).

In order to understand the fate of RGNNV in virus-exposed clams, we optimised a culture assay method using the SSN-1 fish cell line for quantification of viable virus in clam hepatopancreas tissue, faecal matter and water samples. Through this culture assay, we were able to determine whether clams bioaccumulate viable RGNNV after experimental exposure to the virus, and to determine their proficiency to shed viable RGNNV particles into the surrounding environment. Accordingly, in our study molecular detection confirmed NNV-RNA presence in virus-exposed clams, and the optimised culture-based method permitted us to successfully quantify viable virus, thus providing more relevant epidemiological data.

The physiology and morphology of pathogen microorganisms influence the ability of the bivalve molluscs to inactivate or to accumulate and then shed viable microorganisms (Molloy et al. 2013, 2014). Blue mussels *Mytilus edulis* are capable of bioaccumulating finfish viral pathogens, such as ISAV and IPNV. In particular, ISAV is inactivated by *M. edulis*; therefore, viable viral particles are not shed into the water. Conversely, viable IPNV shed by IPNV-exposed mussels may infect cohabitating Atlantic salmon S*almo salar* (Molloy et al. 2013, 2014).

In our study, Manila clams had clearly accumulated viable RGNNV in the hepatopancreas tissue. During the 24 h exposure trials, time did not show a significant effect on the RGNNV load in clam tissues. However, the viral load and the number of positive clams at virus isolation increased progressively during the exposure trials. Significantly, the decrease of viable virus in water during the exposure trials suggests the bioaccumulator role of clams and their ability to remove viable RGNNV from the water column. However, the RGNNV loads in clam tissues were not significantly higher than RGNNV levels in the water, indicating that clams do not concentrate RGNNV in their tissues.

A previous study, observing IPNV uptake by mussels during a 120 h trial, showed that mussels significantly accumulate viable IPNV in their digestive gland tissues over time (Molloy et al. 2013). However, that study also showed that IPNV particles were not efficiently removed from the water column. The authors hypothesised that the small particle size of IPNV (60 nm) may contribute to the inefficiency of particle uptake by the mussel (Molloy et al. 2013). However, bivalve molluscs can concentrate virus as small as RGNNV (25 nm), such as hepatisis A virus (27 nm) (Wolf 1988, Enriquez et al. 1992). Viral uptake and concentration ability of bivalve molluscs can vary from one

virus to another, indicating the presence of different factors contributing to virus uptake (Bosch et al. 1995, Molloy et al. 2013).

RGNNV-exposed clams were able to release viable RGNNV via faecal matter and filtered water. RGNNV was detected in faecal matter and water up to 7 and 4 dpd, respectively. Furthermore, virus detected in the faecal material was significantly higher than that in the water. The finding of higher amounts of RGNNV and its presence for a longer time in faecal matter than in the water suggest that the virus could be attached to suspended particles. This mechanism has been described for several viruses enhancing pathogen transmission, stability and survival (Bitton 1975, Sakoda et al. 1997, Evans et al. 2014).

Moreover, Trial 2 showed the amount of viable virus shed daily by RGNNV-exposed clams into the surrounding environment and in particular in the water until the fourth day and in the faecal matter until the seventh day of depuration, and it showed the persistence of RGNNV in the clam tissue. The shedding by clams of viable RGNNV after daily 100% water changes stresses the persistence of viable virus in hepatopancreas tissues.

This work, together with previous studies of Molloy et al. (2013, 2014) and Skår & Mortensen (2007), suggests that the inactivation of viruses is influenced by their morphology. In particular, non-enveloped viruses such as IPNV and RGNNV can be bioaccumulated by bivalve molluscs and be released alive into the water column (Molloy et al. 2013). In contrast, mussels act as a barrier for enveloped viruses such as ISAV (Molloy et al. 2014). Accordingly, our study, showing the persistence and shedding of viable RGNNV by clams, supports this hypothesis.

The fate of a microbe in bivalve tissue will be determined by a balance between uptake rate, digestion and depuration (Skår & Mortensen 2007). The finding of viable RGNNV shed through faecal matter and filtered water after 1 dpd suggests the potential of some filtered RGNNV particles to bypass the digestive system and be released back into the environment as viable particles entrapped in pseudofaecal pellets, as already hypothesised for other viral particles (Molloy et al. 2013).

The role of clams as bioaccumulators and shedders of viable RGNNV could put at risk susceptible cohabitating fish in an analogous way to that demonstrated by Molloy et al. (2013) for IPNV and Atlantic salmon. However, while virus shed into the water column in a fish farm during an outbreak is diluted by the water current, laboratory challenges are normally performed with high doses of pathogens in static or semi-static systems (Skår & Mortensen 2007). Hence, it is difficult to predict whether wild or cultured clams near farms of susceptible species might act as the causative agents of new outbreaks.

Betanodaviruses, including RGNNV, are very resilient in the aquatic environment, and their presence has already been reported in wild marine invertebrates, especially molluscs and other invertebrates used as live fish food, including *Artemia* sp. nauplii, copepods (*Tigriopus japonicas*) and shrimps (*Acetesinte medius*) (Chi et al. 2003, Gomez et al. 2008b,c, Costa & Thompson 2016). Furthermore, a recent study has shown that trash fish can be a source of betanodaviruses for cultured marine fish (Gomez et al. 2010). Similarly, the presence of natural RGNNV-contaminated invertebrates, including Manila clam, close to susceptible cultured fish species, both in a natural marine environment and in artificial systems (live feed), could behave as RGNNV-reservoirs and be a source of viruses, posing a serious risk of outbreaks of viral nervous necrosis in susceptible cultured fish.

CONCLUSION

The cell culture method set up in this study has improved our understanding of the fate of RGNNV in experimentally challenged Manila clam *Ruditapes philippinarum*. Clams are able to take up and then shed viable RGNNV into the surrounding environment through faeces and filtered water. The persistence of viable RGNNV in clam tissues and the shedding of virus into the surrounding environment present a possible risk for susceptible cohabitant fish species. However, more work should be done in the future in this interesting field to provide more information. Further studies could establish whether the viral transmission from RGNNV-contaminated molluscs to finfish may be a result of viral release into the water or even a result of direct consumption of molluscs by fish. Based on the results of our study, there is little doubt that placing contaminated molluscs into a fish farm, without proper control, could represent a possible risk for farmed fish.

LITERATURE CITED

Bitton G (1975) Adsorption of viruses onto surfaces in soil and water. Water Res 9:473–484 doi:10.1016/0043-1354(75)90071-8

Bosch A, Pinto RM, Abad FX (1995) Differential accumulation and depuration of human enteric viruses by mussels. Water Sci Technol 31:447–451 doi:10.1016/0273-1223(95)00310-J

Chi SC, Shieh JR, Lin SJ (2003) Genetic and antigenic analysis of betanodaviruses isolated from aquatic organisms in Taiwan. Dis Aquat Org 55:221–228 PubMed doi:10.3354/dao055221

Ciulli S, Gallardi D, Scagliarini A, Battilani M, Hedrick RP, Prosperi S (2006) Temperature-dependency of *Betanodavirus* infection in SSN-1 cell line. Dis Aquat Org 68:261–265 PubMed doi:10.3354/dao068261

Ciulli S, Galletti E, Grodzki M, Alessi A, Battilani M, Prosperi S (2007) Isolation and genetic characterization of *Betanodavirus* from wild marine fish from the Adriatic sea. Vet Res Commun 31(Suppl 1):221–224 PubMed doi:10.1007/s11259-007-0010-y

Ciulli S, Volpe E, Pagliarani A, Zavatta E and others (2017) A preliminary study on a novel sea water disinfection process by a peroxy-acid compound to complement and improve the microbial depuration of clams (*Ruditapes philippinarum*). Food Control 80:226–235

Costa JZ, Thompson KD (2016) Understanding the interaction between Betanodavirus and its host for the development of prophylactic measures for viral encephalopathy and retinopathy. Fish Shellfish Immunol 53:35–49 PubMed doi:10.1016/j.fsi.2016.03.033

Doan QK, Vandeputte M, Chatain B, Morin T, Allal F (2017) Viral encephalopathy and retinopathy in aquaculture: a review. J Fish Dis 40:717–742 PubMed

Enriquez R, Froesner GG, Hochstein-Mintzel BV, Riedemann S, Reinhardt G (1992) Accumulation and persistence of hepatitis A virus in mussels. J Med Virol 37:174–179 PubMed doi:10.1002/jmv.1890370305

Evans O, Paul-Pont I, Hick P, Whittington RJ (2014) A simple centrifugation method for improving the detection of Ostreid herpesvirus-1 (OsHV-1) in natural seawater samples with an assessment of the potential for particulate attachment. J Virol Methods 210:59–66 PubMed doi:10.1016/j.jviromet.2014.09.023

Gomez DK, Sato J, Mushiake K, Isshiki T, Okinaka Y, Nakai T (2004) PCR-based detection of betanodaviruses from cultured and wild marine fish with no clinical signs. J Fish Dis 27:603–608 PubMed doi:10.1111/j.1365-2761.2004.00577.x

Gomez DK, Baeck GW, Kim JH, Choresca CH Jr, Park SC (2008a) Molecular detection of betanodavirus in wild marine fish populations in Korea. J Vet Diagn Invest 20:38–44 PubMed doi:10.1177/104063870802000107

Gomez DK, Baeck GW, Kim JH, Choresca CH Jr, Park SC (2008b) Molecular detection of betanodaviruses from apparently healthy wild marine invertebrates. J Invertebr Pathol 97:197–202 PubMed doi:10.1016/j.jip.2007.10.012

374 Gomez DK, Baeck GW, Kim JH, Choresca CH Jr, Park SC (2008c) Genetic analysis of
375 betanodaviruses in subclinically infected aquarium fish and invertebrates. Curr Microbiol 56:499–
376 504 PubMed doi:10.1007/s00284-008-9116-x

377 Gomez DK, Mori KI, Okinaka Y, Nakai T, Park SC (2010) Trash fish can be a source of
378 betanodaviruses for cultured marine fish. Aquaculture 302:158–163
379 doi:10.1016/j.aquaculture.2010.02.033

380 Guo YX, Wei T, Dallmann K, Kwang J (2003) Induction of caspase-dependent apoptosis by
381 betanodaviruses GGNNV and demonstration of protein a as an apoptosis inducer. Virology 308:74–
382 82 PubMed doi:10.1016/S0042-6822(02)00098-3

383 Hierholzer JC, Killington RA (1996) Virus isolation and quantitation. In: Mahy BWJ, Kangro HO
384 (eds) Virology methods manual. Academic Press Limited, London, p 25–46

385 Liu XD, Huang JN, Weng SP, Hu XQ and others (2015) Infections of nervous necrosis virus in
386 wild and cage-reared marine fish from South China Sea with unexpected wide host ranges. J Fish
387 Dis 38:533–540 PubMed doi:10.1111/jfd.12265

388 Molloy SD, Peitrak MR, Bouchard DA, Bricknell I (2011) Ingestion of *Lepeophtheirus salmonis* by
389 the blue mussel *Mytilus edulis.* Aquaculture 311:61–64 doi:10.1016/j.aquaculture.2010.11.038

390 Molloy SD, Pietrak MR, Bricknell I, Bouchard DA (2013) Experimental transmission of infectious
391 pancreatic necrosis virus from the blue mussel, *Mytilus edulis*, to cohabitating Atlantic salmon
392 (*Salmo salar*) smolts. Appl Environ Microbiol 79:5882–5890 PubMed doi:10.1128/AEM.01142-13

393 Molloy SD, Pietrak MR, Bouchard DA, Bricknell I (2014) The interaction of infectious salmon
394 anaemia virus (ISAV) with the blue mussel, *Mytilus edulis.* Aquacult Res 45:509–518
395 doi:10.1111/j.1365-2109.2012.03254.x

396 Mori K, Nakai T, Muroga K, Arimoto M, Mushiake K, Furusawa I (1992) Properties of a new virus
397 belonging to Nodaviridae found in larval striped jack (*Pseudocaranx dentex*) with nervous necrosis.
398 Virology 187:368–371 PubMed doi:10.1016/0042-6822(92)90329-N

399 Mortensen SH (1993) Passage of infectious pancreatic necrosis virus (IPNV) through invertebrates
400 in an aquatic food chain. Dis Aquat Org 16:41–45 doi:10.3354/dao016041

401 Mortensen S (2000) Scallop introductions and transfers, from an animal health point of view.
402 Aquacult Int 8:123–138 doi:10.1023/A:1009282112691

403 Mortensen SH, Bachere E, Le Gall G, Mialhe E (1992) Persistence of infectious pancreatic necrosis
404 virus (IPNV) in scallops *Pecten maximus.* Dis Aquat Org 12:221–227 doi:10.3354/dao012221

405 Mortensen S, Korsnes K, Bergh O (2006) 'Eyes wide shut'—a critical view of aquaculture health
406 management and risk factors in the 'real world'. Bull Eur Assoc Fish Pathol 26:1–5

407 Munday BL, Kwang J, Moody N (2002) Betanodavirus infections of teleost fish: a review. J Fish
408 Dis 25:127–142 doi:10.1046/j.1365-2761.2002.00350.x

409 Nishizawa T, Mori K, Nakai T, Furusawa I, Muroga K (1994) Polymerase chain reaction (PCR)
410 amplification of RNA of striped jack nervous necrosis virus (SJNNV). Dis Aquat Org 18:103–107
411 doi:10.3354/dao018103

412 Noble R, Fuhrman J (1997) Virus decay and its causes in coastal waters. Appl Environ Microbiol
413 63:77–83 PubMed

414 OIE (World Organisation for Animal Health) (2013) Manual of diagnostic tests for aquatic animals.
415 http://www.oie.int/international-standard-setting/aquatic-manual/access-online/

416 Paclibare JO, Evelyn TPT, Albright LJ, Prosperi-Porta L (1994) Clearing of the kidney disease
417 bacterium *Renibacterium salmoninarum* from seawater by the blue mussel *Mytilus edulis*, and the
418 status of the mussel as a reservoir of the bacterium. Dis Aquat Org 18:129–133
419 doi:10.3354/dao018129

420 Panzarin V, Fusaro A, Monne I, Cappellozza E, Patarnello P, Bovo G, Cattoli G (2012) Molecular
421 epidemiology and evolutionary dynamics of betanodavirus in southern Europe. Infect Genet Evol
422 12:63–70 PubMed doi:10.1016/j.meegid.2011.10.007

423 Pietrak MR, Molloy SD, Bouchard DA, Singer JT, Bricknell I (2012) Potential role of *Mytilus*
424 *edulis* in modulating the infectious pressure of *Vibrio anguillarum* 02β on an integrated multi-
425 trophic aquaculture farm. Aquaculture 326–329:36–39 doi:10.1016/j.aquaculture.2011.11.024

426 Sakoda A, Sakai Y, Hayakawa K, Suzuki M (1997) Adsorption of viruses in water environment
427 onto solid surfaces. Water Sci Technol 35:107–114 doi:10.1016/S0273-1223(97)00120-0

428 Sinton L, Hall C, Lynch P, Davies-Colley R (2002) Sunlight inactivation of fecal indicator bacteria
429 and bacteriophages from waste stabilization pond effluent in fresh and saline waters. Appl Environ
430 Microbiol 68:1122–1131 PubMed doi:10.1128/AEM.68.3.1122-1131.2002

431 Skår CK, Mortensen S (2007) Fate of infectious salmon anemia virus (ISAV) in experimentally
432 challenged blue mussels *Mytilus edulis*. Dis Aquat Org 74:1–6 PubMed doi:10.3354/dao074001

433 Thiéry R, Johnson KL, Nakai T, Schneemann A, Bonami JR, Lightner DV (2011) Family
434 *Nodaviridae*. In: King AMQ, Adams MJ, Carstens EB, Lefkowitz EJ (eds) Virus taxonomy. Ninth

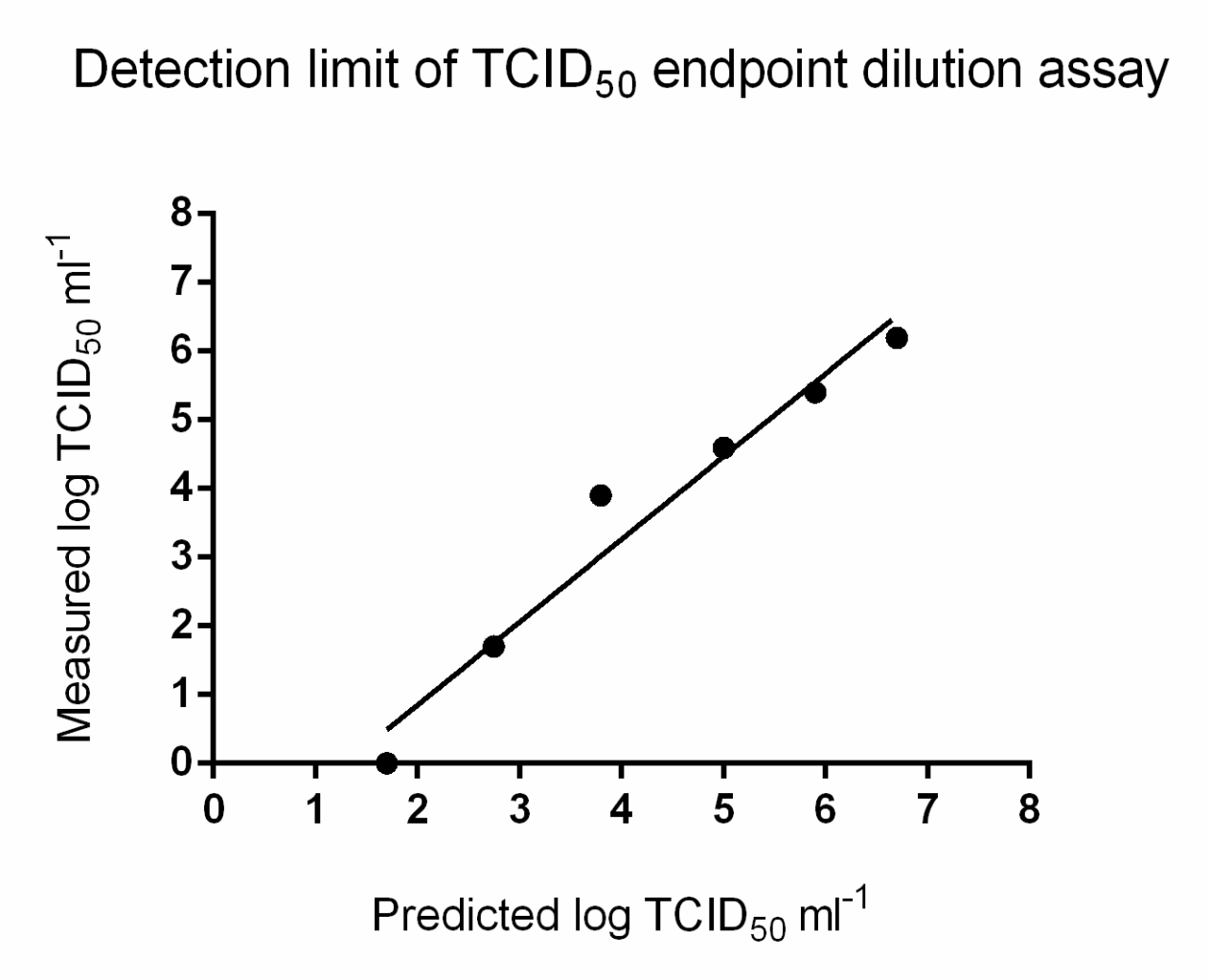
Report of the International Committee on Taxonomy of Viruses. Elsevier Academic Press, San Diego, CA, p 1061–1067

Vendramin N, Patarnello P, Toffan A, Panzarin V and others (2013) Viral encephalopathy and retinopathy in groupers (*Epinephelus* spp.) in southern Italy: a threat for wild endangered species. BMC Vet Res 9:20 PubMed doi:10.1186/1746-6148-9-20

Wangen IH, Karlsbakk E, Einen ACB, Ottem KF, Nylund A, Mortensen S (2012) Fate of *Francisella noatunensis*, a pathogen of Atlantic cod *Gadus morhua*, in blue mussels *Mytilus edulis*. Dis Aquat Org 98:63–72 PubMed doi:10.3354/dao02427

Wilhelm SW, Jeffrey WH, Dean AL, Meador J, Pakulski JD, Mitchell DL (2003) UV radiation induced DNA damage in marine viruses along a latitudinal gradient in the southeastern Pacific Ocean. Aquat Microb Ecol 31:1–8 doi:10.3354/ame031001

Wolf K (1988) Infectious pancreatic necrosis. In: Wolf K (ed) Fish viruses and fish viral diseases. Cornell University Press, Ithaca, NY, p 115-157



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457 Fig. 1. Detection limit of the $TCID_{50}$ endpoint dilution assay, showing a comparison between
458 measured (dots) and predicted (line) log $TCID_{50}$ ml^{-1} of redspotted grouper nervous necrosis virus
459 (RGNNV)-inoculated Manila clam *Ruditapes philippinarum* hepatopancreas homogenates
460 determined in striped snakehead fish cells (SSN-1). Linear regression analysis showed a significant
461 association between measured and predicted values ($p = 0.001$, $R^2 = 0.96$)

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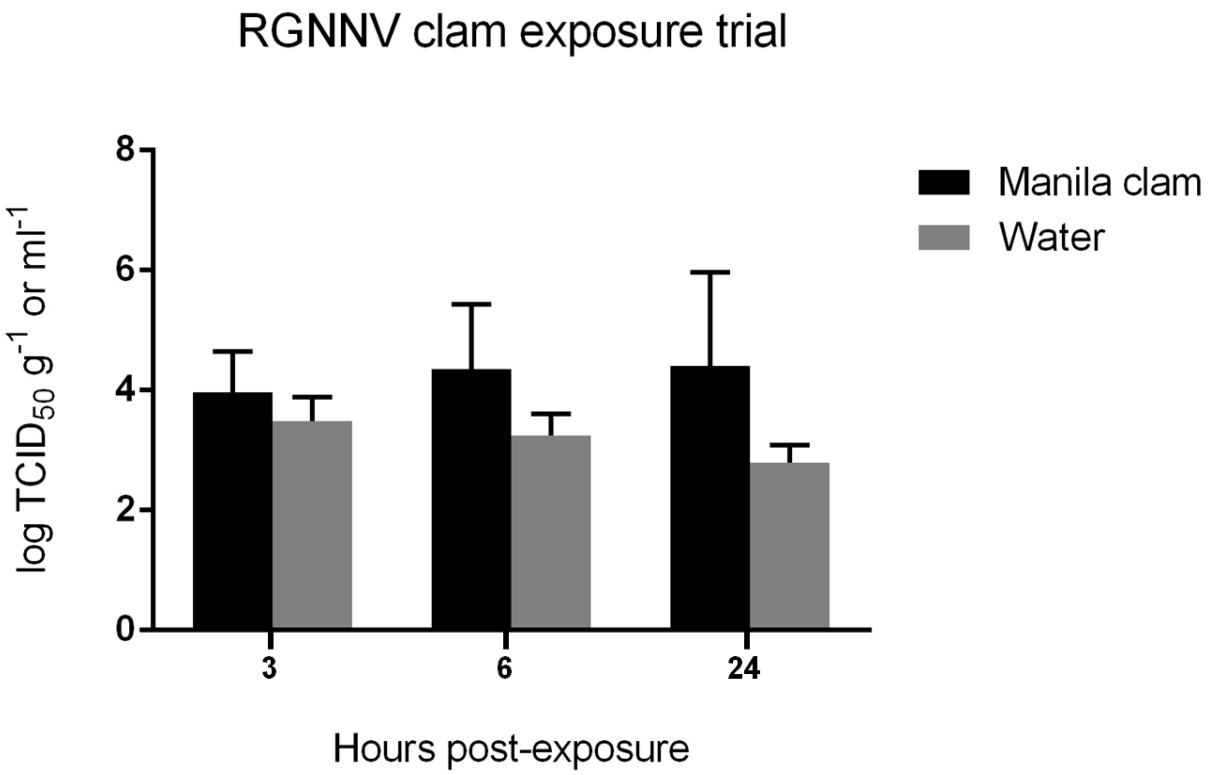

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467 Fig. 2. Exposure of Manila clam *Ruditapes philippinarum* to RGNNV. Bars represent mean ± SD
468 RGNNV loads in clam hepatopancreas and water samples over time. The amount of viable RGNNV
469 increased from 4.0 ± 0.2 to 4.4 ± 0.5 log $TCID_{50}$ g^{-1}. The virus titres detected in water samples
470 decreased from 3.5 ± 0.3 to 2.8 ± 0.2 log$TCID_{50}$ ml^{-1}. No statistical significance was evident

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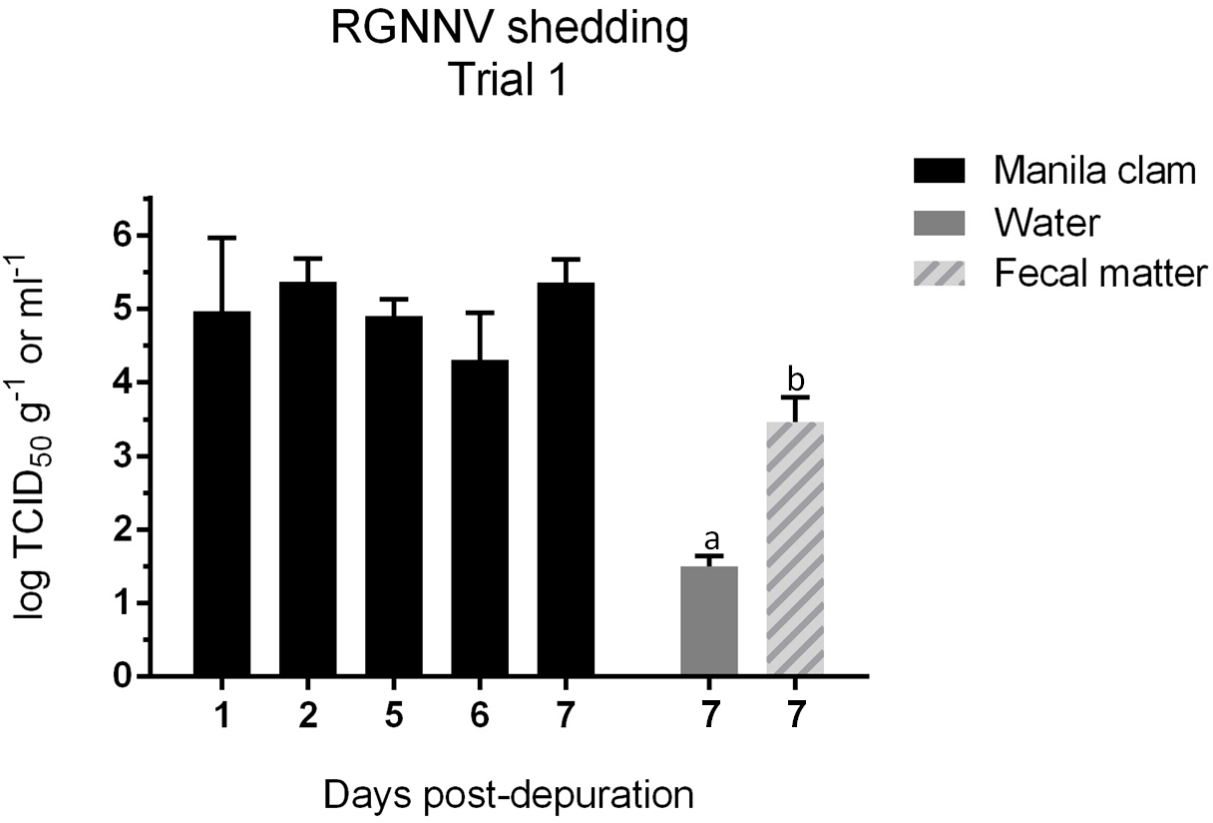

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475 Fig. 3. RGNNV shedding Trial 1. Bars represent the mean ± SD RGNNV loads in Manila clam
476 *Ruditapes philippinarum* hepatopancreas, water and faecal matter samples over time. No significant
477 differences were present among viable RGNNV amounts at different time points in hepatopancreas
478 samples. The titres of viable RGNNV detected in water and faecal matter were statistically lower ($p < 0.05$) than viable RGNNV found in the clam hepatopancreas

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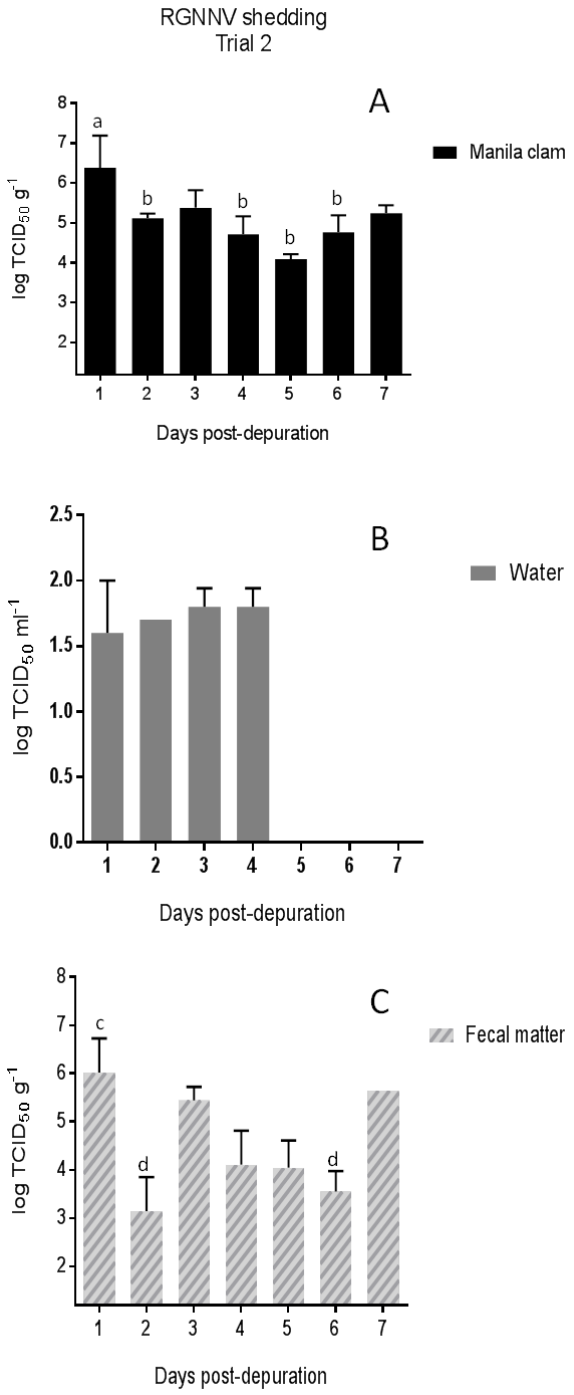

Fig. 4. RGNNV shedding Trial 2. Bars represent the mean ± SD RGNNV loads in (A) Manila clam *Ruditapes philippinarum* hepatopancreas, (B) water and (C) faecal matter samples. Different superscript letters indicate significantly different values. RGNNV was detected in hepatopancreas and faecal matter up to 7 d post-depuration and up to 4 d post-depuration in water. The titrations showed statistically lower values in water samples than in the hepatopancreas and the faecal matter at all tested time points ($p < 0.05$)