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

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

3  
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9 Running Page Head: Volpe et al.: RGNNV in experimentally challenged *Ruditapes philippinarum*

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13  
14 **ABSTRACT:** Redspotted grouper nervous necrosis virus (RGNNV), genus *Betanodavirus*, family  
15 *Nodaviridae*, is the causative agent of viral encephalopathy and retinopathy (otherwise known as  
16 viral nervous necrosis), and can infect several fish species worldwide. Betanodaviruses, including  
17 RGNNV, are very resilient in the aquatic environment, and their presence has already been reported  
18 in several wild marine species including invertebrates. In order to investigate the interaction  
19 between a bivalve mollusc (Manila clam *Ruditapes philippinarum*) and RGNNV, we optimised a  
20 culture-based method. The bioaccumulation of the pathogenic RGNNV by *R. philippinarum* and the  
21 potential shedding of viable RGNNV from RGNNV-exposed clams were evaluated through a  
22 culture-based method. *R. philippinarum* clearly accumulated viable RGNNV in their  
23 hepatopancreatic tissue and were able to release viable RGNNV via faecal matter and filtered water  
24 into the surrounding environment. The role of clams as bioaccumulators and shedders of viable  
25 RGNNV could put susceptible cohabiting cultured fish at risk. RGNNV-contaminated molluscs  
26 could behave as reservoirs for this virus and may modify the virus epidemiology.

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

## 30 INTRODUCTION

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

46 *Betanodavirus* infection is transmitted horizontally, either directly through the introduction of  
47 infected fish, or indirectly by contaminated water and equipment, as well as vertically, through  
48 reproduction (Munday et al. 2002). Recent studies have demonstrated that a certain population of  
49 apparently healthy wild marine fish carry betanodaviruses, and suggested that these wild fish can be  
50 a persistent potential source of virus for cultured fish and the breeding environment (Ciulli et al.  
51 2007, Gomez et al. 2008a, Vendramin et al. 2013). Moreover, a recent finding suggests that trash  
52 fish/molluscs can be a source of betanodaviruses for cultured fish and that they pose a serious risk  
53 for outbreaks of VER in susceptible cultured fish (Gomez et al. 2010). Currently, no successful  
54 therapies or commercial vaccines, apart from one in Japan (OIE 2016), are available to enable  
55 adequate control of VER, so disease prevention is based mainly on maintaining proper sanitary  
56 procedures, screening activities and correct farm management (Costa & Thompson 2016, Doan et  
57 al. 2017).

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

63 onto the surfaces of suspended particles, and uptake in filter-feeding organisms or particle-feeding  
64 plankton (Bitton 1975, Noble & Fuhrman 1997, Sakoda et al. 1997, Skår & Mortensen 2007, Sinton  
65 et al. 2002, Wilhelm et al. 2003, Evans et al. 2014). Accordingly, aquatic organism interaction, both  
66 in the case of natural or artificial environments, such as integrated multi-trophic aquaculture, can  
67 greatly affect the epidemiology of fish infectious diseases. In fact, there is evidence indicating that,  
68 when placed closely together, bivalves may act either as bio-filters or as reservoirs for finfish  
69 pathogens, as a consequence of their ability to bioaccumulate microorganisms (Mortensen et al.  
70 1992, Mortensen 1993, Paclibare et al. 1994, Skår & Mortensen 2007, Molloy et al. 2011, Pietrak et  
71 al. 2012, Wangen et al. 2012). However, the outcome of the interaction may differ on the basis of  
72 the morphology and physiology of the pathogen, which influences whether the pathogen remains  
73 viable in bivalve mollusc tissues and is shed back into the environment, or is inactivated by the  
74 molluscs (Skår & Mortensen 2007, Molloy et al. 2013).

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

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

85

## 86 **MATERIALS AND METHODS**

### 87 **Clam maintenance and NNV screening**

88 Batches of market-size Manila clams, hereafter referred to simply as clams, were obtained from a  
89 commercial clam trader and were reared in an artificial recirculation system (Adriatic Sea  
90 International) supplied with natural seawater, collected from the Adriatic Sea and held at 15°C.  
91 Batches of clams were acclimated for 24 h in order to start filtration. Trials were conducted in a  
92 static system consisting of 5 l plastic tanks supplied with 2 l natural seawater, aerated and held at  
93 15°C.

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

## 102 **Cell culture maintenance and virus propagation**

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

108 The previously characterised RGNNV isolate It/351/Sb (Ciulli et al. 2006) was propagated in SSN-  
109 1 cells grown at 25°C in L-15 medium containing 2% FBS. When the cells demonstrated a 75%  
110 CPE, the cells and supernatant were centrifuged at  $500 \times g$  (10 min), and the supernatant was stored  
111 at –80°C until use. The titre of the stock was determined by 50% tissue culture infectious dose  
112 (TCID<sub>50</sub>) end point analysis in SSN-1 cells. The TCID<sub>50</sub> was calculated according to the Spearman-  
113 Karber method (Hierholzer & Killington 1996).

## 114 **Culture analysis of clam hepatopancreas, faecal matter and water samples**

115 RGNNV presence was detected and quantified by performing TCID<sub>50</sub> analysis in SSN-1 cells in  
116 hepatopancreas tissue, faecal matter and water samples. Water samples were centrifuged at  $3000 \times$   
117  $g$  (5 min) and the supernatant was filtered through 0.20  $\mu\text{m}$  pore size filters and incubated with 1%  
118 antibiotic–antimycotic solution (Gibco) at 4°C overnight. Samples were diluted 10-fold in L-15  
119 with 2% FBS (Gibco). If samples reported negative results, a 2-fold dilution of the supernatants was  
120 performed and tested.

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

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

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

#### 135 **Endpoint dilution assay detection limit in RGNNV-inoculated clam hepatopancreas** 136 **homogenates**

137 Hepatopancreas from 7 NNV-RNA-negative clams were weighed (mean 69.6 ± 23.2 g), diluted 1:9  
138 (wt/vol) with L-15 containing 2% FBS (Gibco) and homogenised before centrifuging at 3000 × g (5  
139 min). Serial 10-fold dilutions of stock RGNNV, ranging in titre from 7.5 to 2.5 log TCID<sub>50</sub> ml<sup>-1</sup>,  
140 were prepared in L-15 cell culture medium. Each virus dilution was added in 100 µl volumes to 6 of  
141 the 7 hepatopancreas homogenates and thoroughly mixed to achieve predicted titres ranging from  
142 6.7 to 1.7 log TCID<sub>50</sub> ml<sup>-1</sup>. L-15 containing 2% FBS was added to the seventh homogenised  
143 sample, which served as a negative control for the TCID<sub>50</sub> assays. RGNNV-inoculated  
144 hepatopancreas homogenates were processed for TCID<sub>50</sub> analysis in SSN-1 as described above.

#### 145 **RGNNV clam exposure trial**

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

## 157 **Clam RGNNV shedding trials**

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

### 160 **Trial 1**

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

### 167 **Trial 2**

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

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

## 176 **Statistics**

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

183

184

185



## 186 **RESULTS**

### 187 **Clam maintenance and NNV screening**

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

### 193 **Endpoint dilution assay detection limit in RGNNV-inoculated clam hepatopancreas** 194 **homogenates**

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

### 205 **RGNNV clam exposure trial**

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

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

217 samples showed no statistical significance; nevertheless, virus titres decreased from  $3.5 \pm 0.3$  to  
218  $2.8 \pm 0.2 \log \text{TCID}_{50} \text{ ml}^{-1}$  (Fig. 2). Molecular detection indicated the presence of NNV RNA in clam  
219 hepatopancreas and water samples at each time point (data not shown).

## 220 **Clam RGNNV shedding trials**

### 221 **Trial 1**

222 Viable RGNNV was isolated from all the clams sampled (Fig. 3). The RGNNV mean titre was  
223  $5.0 \pm 0.2 \log \text{TCID}_{50} \text{ g}^{-1}$ ; we found no statistical significance between viable RGNNV amounts at  
224 different time points in hepatopancreas samples. After 7 dpd, RGNNV-exposed clams released  
225 viable RGNNV into water and through faecal matter (Fig. 3). The titres of viable RGNNV detected  
226 in faecal matter and water were  $3.5 \log \text{TCID}_{50} \text{ g}^{-1}$  and  $1.5 \log \text{TCID}_{50} \text{ ml}^{-1}$ , respectively; these  
227 values were statistically lower ( $p < 0.05$ ) than viable RGNNV found in hepatopancreas tissues ( $5 \pm$   
228  $0.2 \log \text{TCID}_{50} \text{ g}^{-1}$ ). Molecular detection confirmed the presence of NNV RNA in clam  
229 hepatopancreas samples until 7 dpd (data not shown).

### 230 **Trial 2**

231 Viable RGNNV was isolated from all hepatopancreas tissues analysed, with a mean titre of  
232  $5.1 \pm 0.2 \log \text{TCID}_{50} \text{ g}^{-1}$ . RGNNV titre in hepatopancreas at 1 dpd was statistically higher than the  
233 titres at 2, 4, 5 and 6 dpd ( $p < 0.05$ ; Fig. 4A). Viable RGNNV was also isolated from water samples  
234 at 1, 2, 3 and 4 dpd with a mean titre of  $1.3 \pm 0.3 \log \text{TCID}_{50} \text{ ml}^{-1}$ . At 2 dpd, only 1 repeat of the  
235 water sample reported viable RGNNV. No significant differences were revealed among virus titres  
236 detected at different time points (Fig. 4B). In faecal matter, viable RGNNV was isolated from both  
237 repeats of all samples with a mean titre of  $3.9 \pm 0.5 \log \text{TCID}_{50} \text{ g}^{-1}$  except from 1 repeat of the 7 dpd  
238 sample. The titre values in faecal matter samples showed variable amounts of viable RGNNV  
239 during the trial; in particular RGNNV titration at 1 dpd was statistically higher than the titres at 2  
240 and 6 dpd ( $p < 0.05$ ). No significant differences were evident among other time points (Fig. 4C). In  
241 water samples, the titrations showed statistically lower values than in the hepatopancreas tissues and  
242 in the faecal matter samples at all tested time points ( $p < 0.05$ ).

243

## 244 **DISCUSSION**

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

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

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

260 The physiology and morphology of pathogen microorganisms influence the ability of the bivalve  
261 molluscs to inactivate or to accumulate and then shed viable microorganisms (Molloy et al. 2013,  
262 2014). Blue mussels *Mytilus edulis* are capable of bioaccumulating finfish viral pathogens, such as  
263 ISAV and IPNV. In particular, ISAV is inactivated by *M. edulis*; therefore, viable viral particles are  
264 not shed into the water. Conversely, viable IPNV shed by IPNV-exposed mussels may infect  
265 cohabitating Atlantic salmon *Salmo salar* (Molloy et al. 2013, 2014).

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

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

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

283 RGNNV-exposed clams were able to release viable RGNNV via faecal matter and filtered water.  
284 RGNNV was detected in faecal matter and water up to 7 and 4 dpd, respectively. Furthermore, virus  
285 detected in the faecal material was significantly higher than that in the water. The finding of higher  
286 amounts of RGNNV and its presence for a longer time in faecal matter than in the water suggest  
287 that the virus could be attached to suspended particles. This mechanism has been described for  
288 several viruses enhancing pathogen transmission, stability and survival (Bitton 1975, Sakoda et al.  
289 1997, Evans et al. 2014).

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

295 This work, together with previous studies of Molloy et al. (2013, 2014) and Skår & Mortensen  
296 (2007), suggests that the inactivation of viruses is influenced by their morphology. In particular,  
297 non-enveloped viruses such as IPNV and RGNNV can be bioaccumulated by bivalve molluscs and  
298 be released alive into the water column (Molloy et al. 2013). In contrast, mussels act as a barrier for  
299 enveloped viruses such as ISAV (Molloy et al. 2014). Accordingly, our study, showing the  
300 persistence and shedding of viable RGNNV by clams, supports this hypothesis.

301 The fate of a microbe in bivalve tissue will be determined by a balance between uptake rate,  
302 digestion and depuration (Skår & Mortensen 2007). The finding of viable RGNNV shed through  
303 faecal matter and filtered water after 1 dpd suggests the potential of some filtered RGNNV particles  
304 to bypass the digestive system and be released back into the environment as viable particles  
305 entrapped in pseudofaecal pellets, as already hypothesised for other viral particles (Molloy et al.  
306 2013).

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

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

324

## 325 CONCLUSION

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

336

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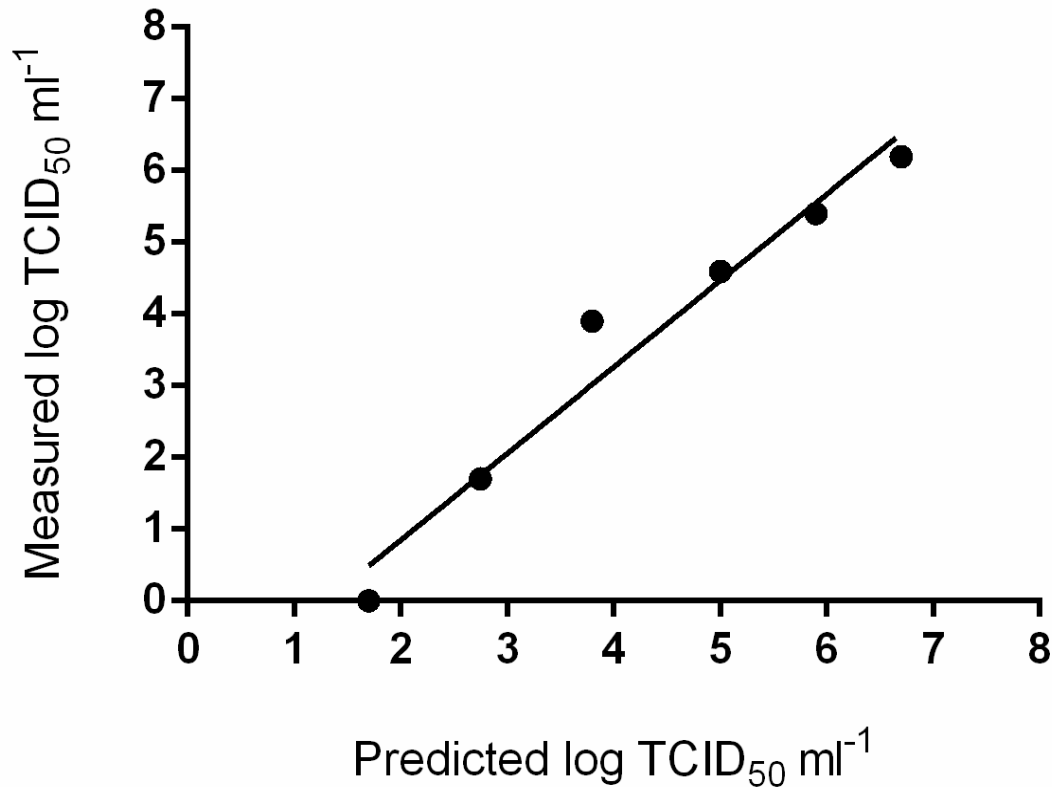
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## Detection limit of TCID<sub>50</sub> endpoint dilution assay



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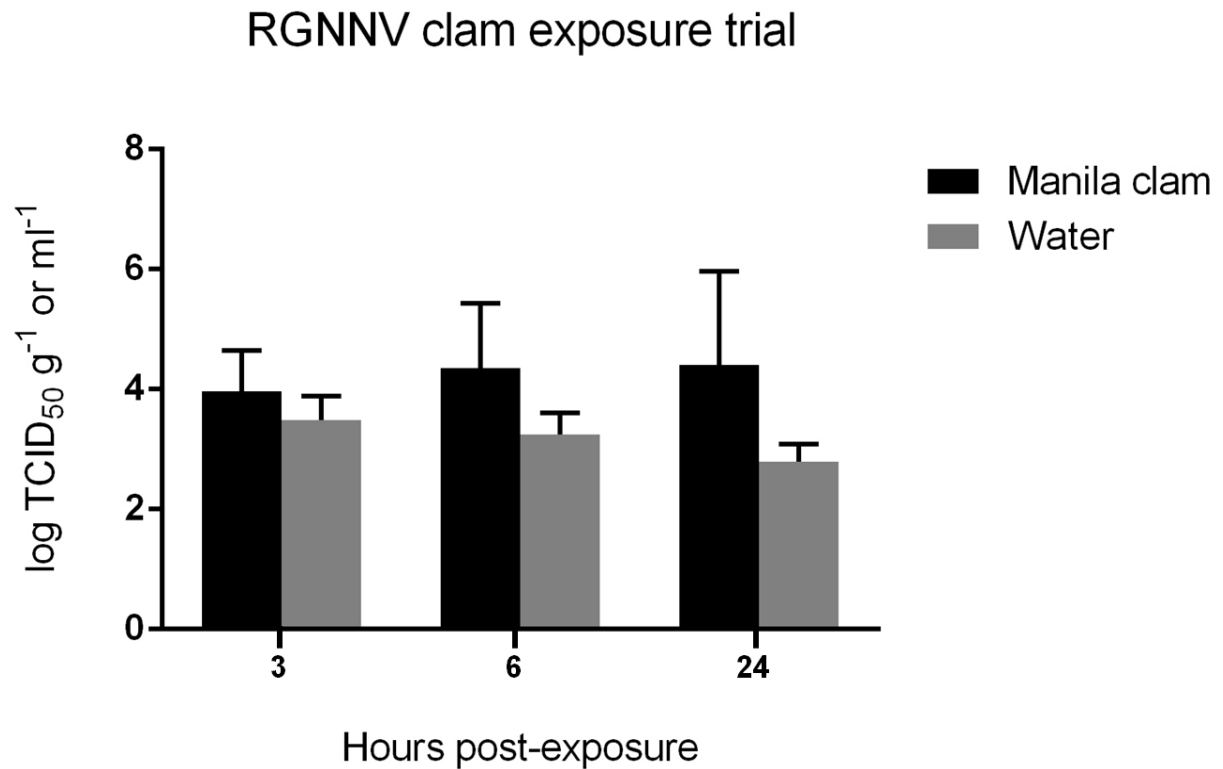
457 Fig. 1. Detection limit of the TCID<sub>50</sub> endpoint dilution assay, showing a comparison between  
458 measured (dots) and predicted (line) log TCID<sub>50</sub> ml<sup>-1</sup> of redspotted grouper nervous necrosis virus  
459 (RGNNV)-inoculated Manila clam *Ruditapes philippinarum* hepatopancreas 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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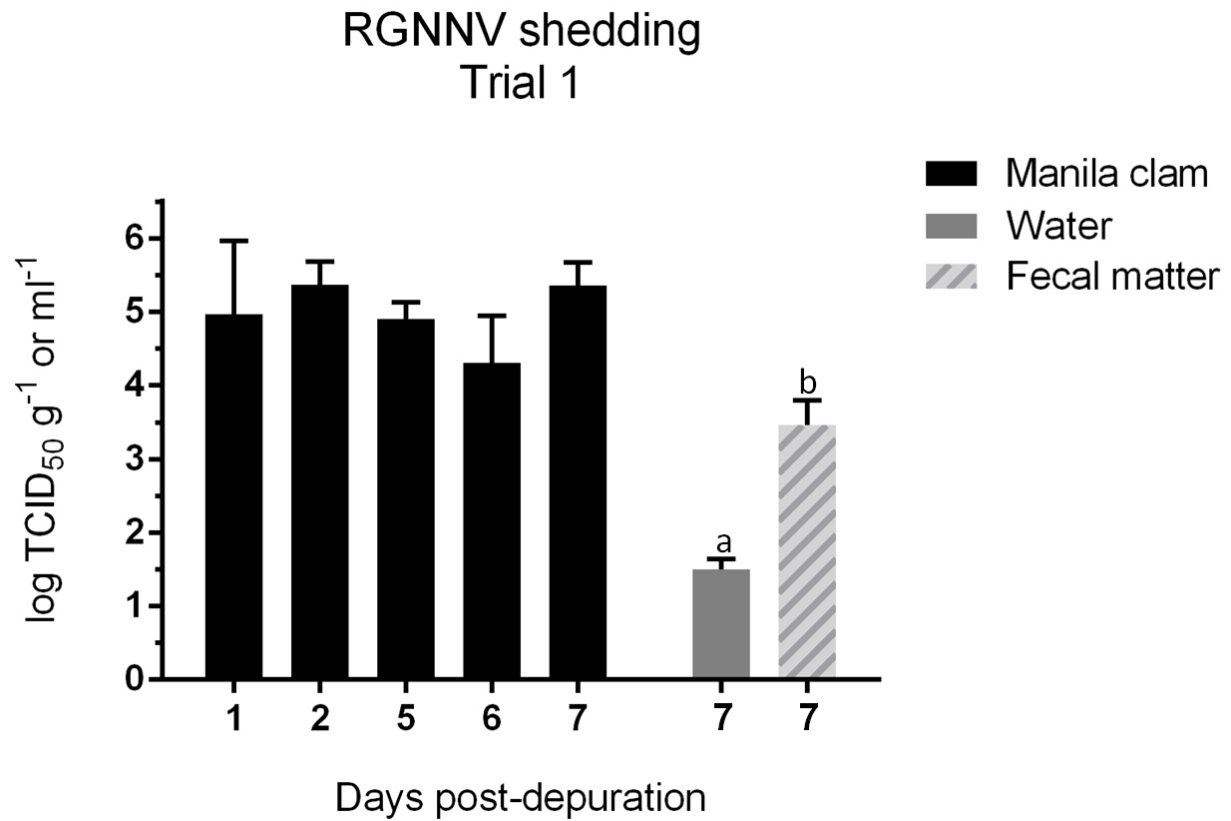


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467 Fig. 2. Exposure of Manila clam *Ruditapes philippinarum* to RGNNV. Bars represent mean ± SD  
 468 RGNNV loads in clam hepatopancreas and water samples over time. The amount of viable RGNNV  
 469 increased from 4.0 ± 0.2 to 4.4 ± 0.5 log TCID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

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475 Fig. 3. RGNNV shedding Trial 1. Bars represent the mean  $\pm$  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 ( $p$   
 479  $< 0.05$ ) than viable RGNNV found in the clam hepatopancreas

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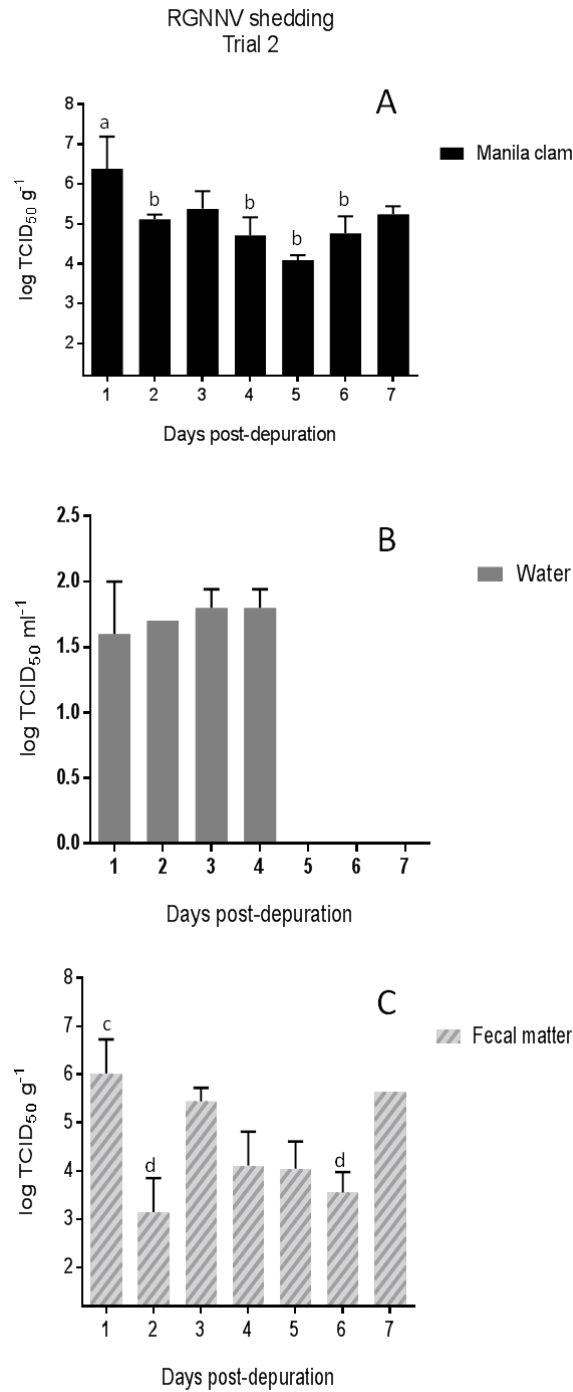


Fig. 4. RGNNV shedding Trial 2. Bars represent the mean  $\pm$  SD RGNNV loads in (A) Manila clam *Ruditapes philippinarum* hepatopancreas, (B) water and (C) faecal matter samples. Different superscript letters indicate significantly different values. RGNNV was detected in hepatopancreas and faecal matter 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 ( $p < 0.05$ )