Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN
Scienze Veterinarie

Ciclo XXIX

Settore Concorsuale di afferenza: 07/H3 – MALATTIE INFETTIVE E PARASSITARIE DEGLI ANIMALI

Settore Scientifico disciplinare: VET/05 – MALATTIE INFETTIVE DEGLI ANIMALI DOMESTICI

TITOLO TESI

Finfish and human pathogens in bivalve molluscs

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Esame finale anno 2017

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Preamble

Bivalve molluscs are an important food source for living beings, including humans. Aquaculture provides 89.6% of global bivalve mollusc production, which plays an important role in ensuring food and employment to the world population (FAO 2013).

Bivalve molluscs are obligated filter feeders, that feed on microalgae, bacteria and organic particles present in the aquatic environment. Accordingly, they could accumulate chemical compounds, marine biotoxins, bacteria and viruses, including human and animal pathogens (Molloy et al., 2013; Serratore et al., 2014).

The interaction between microorganisms and bivalve molluscs, both in natural and artificial environments, such as integrated multitrofic aquaculture, might influence the epidemiology of animal and human infectious diseases (Skår & Mortensen, 2007).

Some studies, investigating the interaction between bivalve molluscs and fish pathogens, show that *Infectious salmon anemia virus* (ISAV) is inactivated from Atlantic mussels (*Mytilus edulis*) (Molloy et al. 2014), whereas *Infectious pancreatic necrosis virus* (IPNV) could be transmitted from contaminated Atlantic mussels to Atlantic salmon (*Salmo salar*) (Molloy et al. 2013).

Previous studies, performed by the virology research group of the DIMEVET, have shown a wide presence of betanodaviruses in bivalve molluscs including Manila clam (*Ruditapes philippinarum*), mussels (*Mytilus galloprovincialis*) and oysters (*Crassostrea gigas*) (Ciulli et al., 2010). Betanodaviruses are small ssRNA viruses of the genus *Betanodavirus*, family *Nodaviridae* (Thiéry et al., 2012) responsible of viral encephalopathy and retinopathy (VER), otherwise known as viral nervous necrosis (VNN), one of the most threatened disease in marine aquaculture worldwide.

The Ph.D thesis, arranged in three chapters, deals with finfish and human pathogens in bivalve molluscs and focus on betanodavirus presence in bivalve molluscs, on their interaction with the *Redspotted grouper nervous necrosis virus* (RGNNV), a viral species of the genus *Betanodavirus*, and the development of a novel method to mitigate bacterial and viral contaminations of bivalve molluscs.

The first chapter reports a research on the molecular detection and phylogenetic analysis of betanodaviruses in bivalve molluscs collected from 2008 to 2015, different European countries and three species. In this study, detected viruses have been analyzed genetically to find out whether bivalve molluscs could be a source of genetically close related betanodaviruses to finfish. In fact, the finding of betanodaviruses in bivalve molluscs strictly related to finfish betanodaviruses could pose a possible risk of inter-specific transmission.

The second chapter focused on the fate of RGNNV in experimentally challenged Manila clam to investigate the potential role of clams as an RGNNV reservoir and the potential risks posed by RGNNV-contaminated molluscs.

The third study deals with sea water disinfection to complement and improve the microbial depuration of Manila clams. A novel sea water disinfection process was tested on Manila clam by employing a potassium peroxymonosulfate (MPS)-based product. The biocidal activity of the disinfection process was evaluated against both bacterial and viral contaminants of bivalve molluscs. Particularly, bactericidal activity was evaluated against the *Vibrio* spp. population naturally associated with sea water that include several human and finfish pathogens; virucidal activity was assessed against VNNV, the most threatening among the viral pathogens of marine finfish.

CHAPTER 1

Molecular detection and phylogenetic analysis of betanodaviruses in bivalve molluscs

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1.1 ABSTRACT

Betanodaviruses are small ssRNA viruses of the family *Nodaviridae* responsible of viral encephalopathy and retinopathy (VER) otherwise known as viral nervous necrosis (VNN) in marine fishes worldwide. They can be transmitted both vertically and horizontally and invertebrates, where they have been detected sporadically, have been suspected to be a source of the virus.

This is the first study focusing on betanodavirus in bivalve molluscs. Twenty seven new betanodavirus strains were detected in bivalve molluscs of different species, European countries and year of collection and genetically characterized.

Betanodaviruses detected in mollusc bivalve and in finfish are very closely related to betanodaviruses previously detected in finfish in Southern Europe from 2000 to 2009. However, also a new betanodavirus strain not belonging to any of the already known betanodavirus genotypes was detected.

Such a massive and variegate presence of betanodaviruses in bivalve molluscs greatly stresses the risks of transmission previously feared for other invertebrates. Molluscs bivalve reared in the same area of farmed and wild finfish could act as a reservoir of virus. Furthermore, the marketing of alive bivalve mollusc and the relaying activity, allowed by the European regulation, can pose also a real risk of spreading betanodavirus between different geographical areas.

1.2 INTRODUCTION

Betanodaviruses are small ssRNA viruses of the genus *Betanodavirus*, family *Nodaviridae* (Thiéry et al., 2012) responsible of viral encephalopathy and retinopathy (VER), otherwise known as viral nervous necrosis (VNN), in several fish species worldwide. Betanodavirus genome consist of two segments named RNA 1 (3.1 KB) and RNA 2 (1.4 kb) coding respectively for the RNA-dependent RNA polymerase and the coat protein. Moreover, during virus replication, a subgenomic transcript called RNA3 is originated from the 3' terminus of RNA1 (Iwamoto et al., 2005; Thiéry et al., 2012). Based on phylogenetic analysis of the T4 variable region within the RNA 2 segment, betanodaviruses have been historically divided into four genotypes, currently accepted as official species of this genus: *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) (Nishizawa et al. 1997; Thiéry et al., 2012). Demarcation of species is mainly based on genetic characterization, however, more genotypes than that recognized as species has been described such in the case of Atlantic cod nervous necrosis virus (ACNNV) (Gagné et al., 2004) and the turbot nodavirus (TNV) (Johansen et al., 2004). Although, genotyping was based on RNA 2 phylogenetic analysis (Nishizawa et al. 1997), RNA 1 phylogenetic analysis added further information showing the presence of reassortant strains (Olveira et al., 2009; Toffolo et al., 2007). As a matter of fact, the presence of reassortant betanodaviruses SJNNV/RGNNV has been previously described in sea bass (*Dicentrarchus labrax*) caught in Italy and Croatia, in the form of a genetic variant containing the RNA 1 segment deriving from the SJNNV genotype and the RNA 2 molecule originating from the RGNNV-type (Toffolo et al., 2007). Later on, a new reassorted betanodavirus, in the form of a RGNNV/SJNNV genetic variant, has been detected in sea bream (*Sparus aurata*), common sole (*Solea solea*) and Senegalese sole (*Solea senegalensis*) farmed in Portugal, Italy and Spain (Olveira et al., 2009; Panzarin et al., 2012).

Viral encephalopathy and retinopathy is observed mainly in farmed fish, however severe outbreaks were observed in wild fish affecting mainly groupers (Gomez et al., 2009; Vendramin et al., 2013). Furthermore, asymptomatic betanodavirus infection is often detected in wild fish (Barker et al., 2002; Gomez et al., 2004; Baeck et al., 2007; Ciulli et al., 2007a; Gomez *et al.* 2008a; Panzarin et al., 2012; Liu et al., 2015). Moreover. sporadic presence of betanodaviruses in invertebrates was shown in the Mediterranean Sea, South Korea and Japan (Gomez et al., 2006; Gomez et al., 2008b; Gomez et al. 2010; Ciulli et al. 2010; Panzarin et al., 2012; Fichi et al., 2015). Particularly, considering bivalve mollusc betanodavirus presence was reported in two mussels (*Mytilus galloprovincialis*) collected in Korea and one clam (*Ruditapes philippinarum*) in Italy (Gomez et al., 2008b; Panzarin et al., 2012). We evidenced betanodaviruses in Italian clams and French oysters

since 2010 and some preliminary results were presented to the 14th International Biotechnology Symposium and Exhibition (Ciulli et al., 2010).

Actually, most of the genetically characterized betanodaviruses detected in bivalve mollusc/invertebrates were included in RGNNV genotype. However, a reassortant RGNNV/SJNNV strain was found in *Artemia salina* and *Opistobranchia* (Gomez et al., 2008b; Gomez et al., 2008c; Ciulli et al., 2010; Panzarin et al., 2012). Overall, a very limited number of studies have been made on this topic. Betanodavirus can be transmitted by both vertical and horizontal transmission. In addition, interspecies transmission is possible and genetically related viruses are often detected in different species. For these reasons wild fish have been supposed reservoir for the virus (Gomez et al., 2006; Gomez et al., 2008a; Doan et al., 2016). Similarly, it was hypothesized that Betanodavirus can be transmitted to finfish trhough trash fish composed of both marine vertebrates and invertebrates (Gomez et al., 2010). However, several factors can affect real risk of betanodavirus transmission from invertebrate to finfish, including the prevalence of the virus in invertebrates populations and the similarity of the virus found in invertebrates with those of finfish. In this study, we examined bivalve molluscs reared in different European countries for the presence of betanodaviruses; detected viruses have been analyzed genetically to find out whether these animals could be a source of genetically close related betanodaviruses to finfish. The finding of betanodaviruses in molluscs strictly related to virus found in fish could pose a possible risk of spreading the virus into new areas.

1.3 MATERIALS AND METHODS

1.3.1 Bivalve mollusc and viruses

Betanodaviruses characterized in this study were obtained through a preliminary survey conducted in 2009 to investigate the betanodavirus presence in three bivalve mollusc species (Ciulli et al., 2010). In the survey, a total of 57 lots (19 for each species) of retail bivalve molluscs were analyzed including a species reared on the seabed such as clam (*Ruditapes philippinarum*) and species usually farmed in the water column such as oysters (*Crassostrea gigas*) and mussels (*Mytilus galloprovincialis*). Each species was equally represented in the sampling. Each sample was composed of 30 clams, 10 mussels or 6 oysters. Bivalve mollusc lots were collected directly from the market in 2009 and originated from France (oysters), Italy (clams and mussels) and Spain (mussels). This survey allowed to collect one betanodavirus strain from mussel, six from oysters and eight from clams (Table 1).

Further diagnostic activity focused on Italian clam was conducted from September 2012 to May 2015 consisting in the betanodavirus screening of further 36 lots of clams. This activity allowed to collect further 12 betanodavirus strains. A strain detected in mussels collected in Sicily in 2008 at the IZS (Istituto Zooprofilattico Sperimentale della Sicilia) was also included in the analysis (Table 1).

Sample names	Species	Years of sampling	Origin
PA3M	mussel	2008	Italy (Sicily)
681M	mussel	2009	Not available
585O, 651O, 664O, 666O, 672O, 686O	oyster	2009	France (Atlantic Ocean)
628C, 629C, 651C, 667C, 671C, 676C, 680C, 684C	clams	2009	Italy (Northern Adriatic)
919C	clams	2012	Italy (Northern Adriatic)
76C, 79C, 133C, 134C, 135C, 229C, 271C, 272C	clams	2014	Italy (Northern Adriatic)
38C, 39C, 58C	clams	2015	Italy (Northern Adriatic)

Table 1. Details of betanodavirus strains detected in bivalve molluscs and used for phylogenetic analysis.

1.3.2 RNA extraction, RT-PCR and nested PCR

The mollusc hepatopancreas were homogenized and treated with proteinase K (Sigma, St. Louis, USA), then RNA was extracted according to the manufacturer's instructions with NucleoSpin® RNA II (Macherey-Nagel, Düren, Germany). RNA samples were stored at -80 °C until use.

Betanodavirus presence was investigated by an RT-nested PCR method using primers previously described targeting the viral RNA 2 (Ciulli et al., 2007b). Briefly, the first amplification step was conducted through a one-step RT-PCR assay with primers S6-S7 (Ciulli et al., 2006) using the SuperScript III One-Step RT-PCR System (Invitrogen, Carlsbad, USA). The reaction mixture consisted of 15 µl of reaction mix containing 3 µl RNA, 7.5 µl 2X Reaction Mix, 0.8 µM of each primer and 0.3 µl Superscript III/Platinum Taq enzyme mix. The optimal thermal cycling conditions were 45 °C for 30 min, 95 °C for 2 min, followed by 40 cycles of 94 °C for 60 s, 58 °C for 60 s and 72 °C for 60 s. A final extension was performed at 72°C for 7 min. Nested PCR was conducted with primers F2-R3 (Nishizawa et al., 1994) using the Platinum Taq DNA polymerase (Invitrogen). The reaction mixture for the nested PCR had a total volume of 25 µl and contained 2.5 µl of 10X PCR buffer, 1.5 mM $MgCl_2$, 0.25 µM of each primer, 1 µl of cDNA diluted 1:100, 1.25 units of Platinum Taq DNA polymerase (Invitrogen) and nuclease free water. The thermal cycle for nested PCR consisted of 95°C for 5 min and 40 cycles of 94°C for 30 sec, 56°C for 30 sec and 72°C for 30 sec. A final extension was performed at 72°C for 7 min. To avoid cross contamination, negative controls were run along with all reactions. The results of all RT-PCR and nested PCR analyses were checked by agarose gel electrophoresis of PCR products along with a 100 bp DNA molecular marker (Invitrogen, Carlsbad, USA).

Nine betanodaviruses detected between 2012 and 2015 were further analyzed for an RNA 1 fragment using primers previously described (Toffolo et al., 2007). RT-PCR was performed with the SuperScript III One-Step RT-PCR System (Invitrogen, Carlsbad, USA) using primers VNNV 5-6. Eminested PCR was conducted with primers VNNV 6-7 using the Platinum Taq DNA polymerase (Invitrogen).

1.3.3 Sequencing and phylogenetic analysis

PCR products were purified using the High Pure PCR Product Purification Kit (Roche, Mannheim, Germany) and then sequenced through the Bio-Fab Sequencing Service (Rome, Italy). Amino acid sequences were predicted by the BioEdit software (http://bioedit.software.informer.com/). Sequences were then aligned and compared with betanodavirus sequences previously obtained from

strains isolated from farmed and wild finfish and with betanodavirus reference genotypes strains (Thiery et al., 2012) available in GenBank (www.ncbi.nlm.nih.gov) using Clustal W in BioEdit software (http://bioedit.software.informer.com/). Particularly, a selection of betanodavirus sequences collected in southern Europe and used for a previous extensive and comprehensive phylogenetic study was used (Panzarin et al., 2012). Percentage of similarity of pairwise distances was calculated with BioEdit software. Phylogenetic analysis was carried out using the MEGA version 6 software (www.megasoftware.net) and employing the general time-reversible (GTR) model of nucleotide substitution. A phylogenetic tree was constructed using the maximum-likelihood method. Bootstrap analysis was carried out on 1000 replicates.

1.4 RESULTS

1.4.1 Virus detection

All samples collected in the 2009 survey resulted negative to RT-PCR, while 15 lots out of 57 resulted positive to nested PCR (26.3 %). Only one positive lot was found out of 19 lots of mussels (*M. galloprovincialis)*, whereas clams (*T. philippinarum*) and oysters (*C. gigas*) resulted highly positive to betanodavirus, with 42.1 % (8/19) and 31.6% (6/19) of lots positive to betanodavirus respectively. All the positives except one (585/2009) were collected from June to September.

1.4.2 Sequencing and phylogenetic analysis

The maximum likelihood phylogenetic trees inferred for the RNA 1 and RNA 2 genes of the viruses collected from shellfish from 2008 to 2015 revealed that all betanodaviruses detected in this study except one (681M/2009) felt within RGNNV genotype (Figs. 1 and 2). The virus 681M/2009 clustered in a group by itself, outside all RGNNV subgroups (Fig. 2). Unfortunately, for this virus, as well as for all viruses detected before 2012 was not possible to carry out RNA 1 sequencing due to the failure storing of samples.

The topology of the RNA 1 and RNA 2 phylogenetic trees confirmed the genetic clustering obtained in a previous study that analyze several betanodavirus sequences collected in southern Europe (Panzarin et al., 2012) (Figs. 1 and 2) therefore corroborating the validity of the analysis based on the partial fragment of RNA 1 (position 678-1097 of SJNNV AB056571) and the T4 variable region of the RNA 2 (position 635-923 of SJNNV AB056572).

RNA 1 and RNA 2 phylogenetic trees identified 11 and 7 well supported monophyletic genetic subgroups inside RGNNV genotype (bootstrap values >70%) (Figs. 1 and 2). For a better comparison clusters were designated with names used in the study of Panzarin et al. (2012).

Analysis of RNA 1 showed that betanodaviruses detected in Italian clams clustered in 3 RGNNV subgroups previously identified and named II, IV and X (Panzarin et al., 2012). On the basis of the comparison with viruses previously included in these groups, some of the 2014 and 2015 clam samples clustered with viruses isolated several years ago (1996-2000) from finfish (cluster X). Clam viruses clustered together with both Italian and other European countries viruses, regardless of host origin, farmed or wild status.

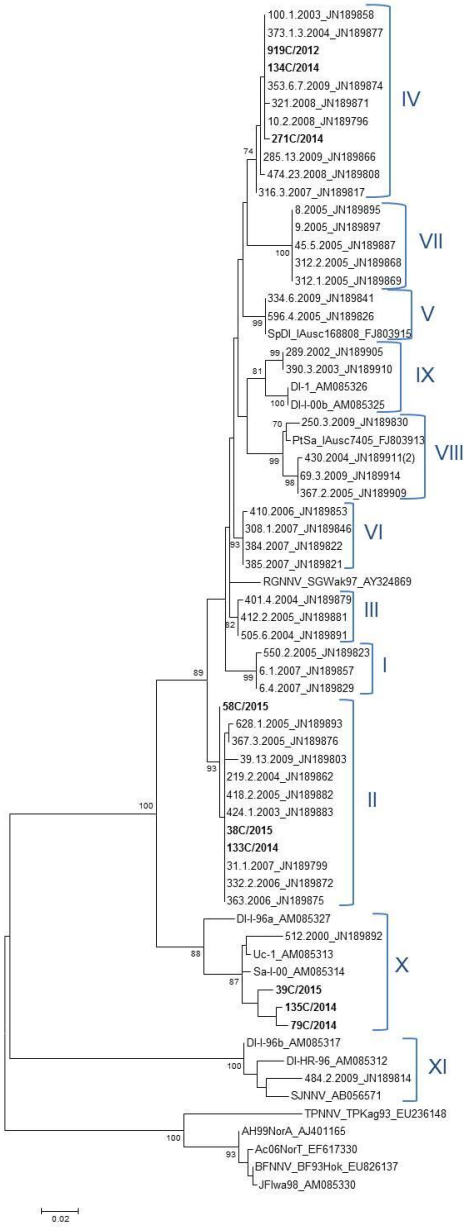

Fig. 2. Phylogenetic tree constructed with RNA 1 fragment of betanodaviruses detected in bivalve molluscs combined with reference strains and finfish betanodavirus sequences retrieved from Genbank.

Analysis of RNA 2 showed that betanodaviruses detected in bivalve molluscs clustered in 2 subgroups (B, E) out of the seven previously identified (Panzarin et al., 2012). Both subgroups included viruses with high variability respect to year of detection and host status (wild/farmed). Moreover, subgroup E included viruses isolated in different European countries, including a virus detected in a French oyster. Two further subgroups were identified in RNA 2 phylogenetic tree. One subgroup (named H) was represented by viruses detected in four French oysters and in one Italian mussel (Sicily). They clustered together with a wild Italian *Epinephelus spp*. virus that did not fall in any subgroups in the previous analysis (Panzarin et al., 2012). The other subgroup (named I) consists of betanodavirus strains detected in seven Italian clams in 2014 and 2015 and European viruses detected in farmed finfish that did not fall in any subgroups in the previous analysis (Panzarin et al., 2012). All genetic clusters were well supported by bootstrap analysis (bootstrap values >70%).

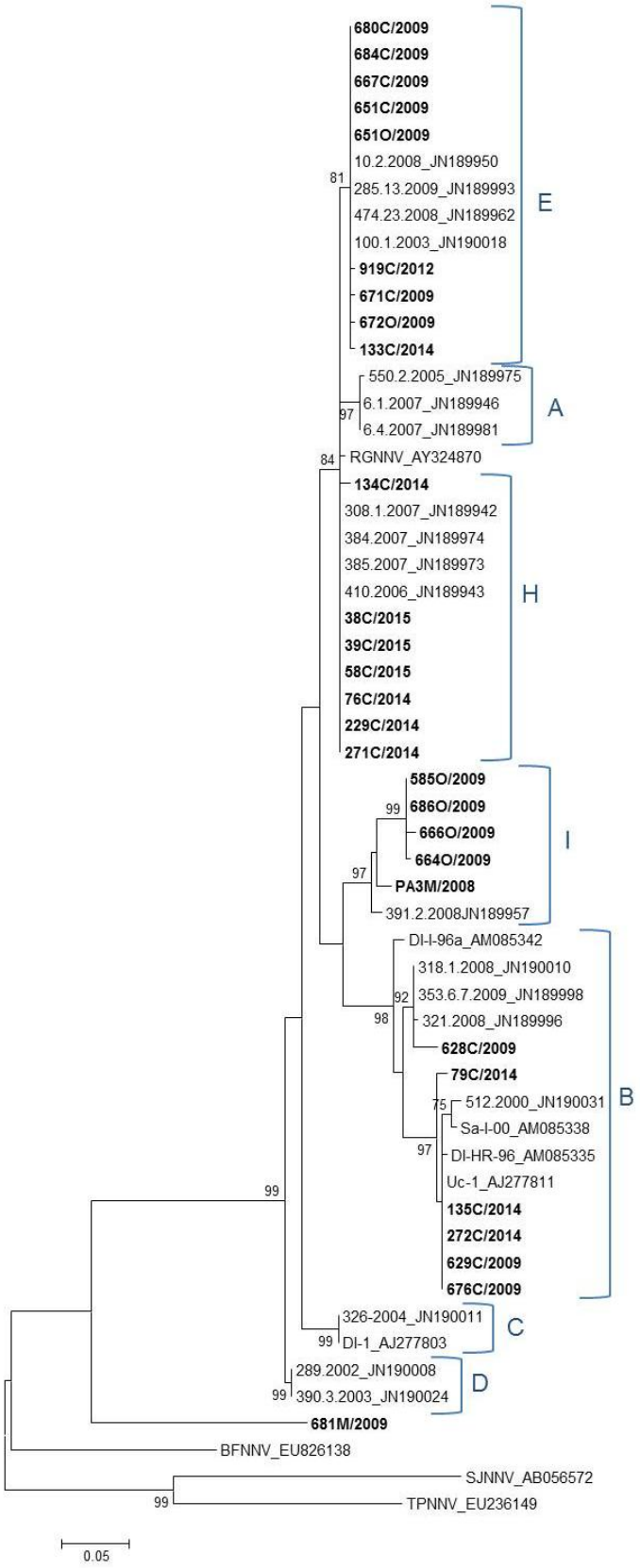

Fig. 3. Phylogenetic tree constructed with RNA 2 fragment of betanodaviruses detected in bivalve molluscs combined with reference strains and finfish betanodavirus sequences retrieved from Genbank.

Betanodaviruses detected in bivalve mollusc in this study showed nucleotide and amino acid identities between each other higher than 88.9% and 86.0% respectively except with strain 681M/2009 that has a nucleotide and amino acid identity always lower than 75.0% and 81.7% respectively.

The percentage of pairwise nucleotide and aminoacid similarity with the RNA 2 of the four betanodavirus genotypes are reported in Table 2.

All the viruses detected in bivalve molluscs except strain 681M/2009 showed the highest nucleotide and amino acid identities with RGNNV genotype ranging between 89.6 and 99.6% and between 87.0 % and 100.0% respectively, whereas the nucleotide identities with other genotypes were lower than 76.8, 65.8 and 65.5% for BFNNV, SJNNV and TPNNV respectively and the amino acid identity was lower than 84.9, 68.4 and 70.5% for BFNNV, SJNNV and TPNNV respectively. Strain 681M/2009 showed similar nucleotide identities with RGNNV (75.0% nucleotide identity and 80.6% amino acid identity) and BFNNV (72.2% nucleotide identity and 79.5% amino acid identity) genotype, these values were lower than those between RGNNV and BFNNV genotypes (76.1% nucleotide identity and 84.9% amino acid identity).

	RGNNV		BFNNV		SJNNV		TPNNV	
	nt	aa	nt	aa	nt	aa	nt	aa
38C/2015	99.6	100	76.5	84.9	65.5	67.3	64.8	69.4
39C/2015	99.6	100	76.5	84.9	65.5	67.3	64.8	69.4
58C/2015	99.6	100	76.5	84.9	65.5	67.3	64.8	69.4
76C/2014	99.6	100	76.5	84.9	65.5	67.3	64.8	69.4
79C/2014	91.1	89.2	71.8	82.7	64.4	68.4	64.8	70.5
133C/2014	98.5	100	76.8	84.9	65.5	67.3	64.8	69.4
134C/2014	98.9	98.9	76.1	83.8	65.1	66.3	64.4	68.4
135C/2014	90.7	89.2	71.5	82.7	65.1	68.4	64.8	70.5
229C/2014	99.6	100	76.5	84.9	65.5	67.3	64.8	69.4
271C/2014	99.6	100	76.5	84.9	65.5	67.3	64.8	69.4
272C/2014	90.7	89.2	71.5	82.7	65.1	68.4	64.8	70.5
919C/2012	98.5	100	76.8	84.9	64.8	67.3	64.8	69.4
585O/2009	93.2	92.4	72.9	81.7	64.4	64.2	60.6	66.3
628C/2009	89.6	87	71.1	79.5	64.8	68.4	63.7	69.4
629C/2009	90.7	89.2	71.5	82.7	65.1	68.4	64.8	70.5
651O/2009	98.9	100	76.5	84.9	65.1	67.3	65.1	69.4
651C/2009	98.9	100	76.5	84.9	65.1	67.3	65.1	69.4
664O/2009	92.8	91.3	72.5	80.6	64.1	63.1	60.6	65.2
666O/2009	92.5	91.3	72.2	81.7	64.4	64.2	60.6	65.2
667C/2009	98.9	100	76.5	84.9	65.1	67.3	65.1	69.4
671C/2009	98.5	100	76.1	84.9	65.5	67.3	64.8	69.4
672O/2009	98.5	100	76.8	84.9	64.8	67.3	65.5	69.4
676C/2009	90.7	89.2	71.5	82.7	65.1	68.4	64.8	70.5
680C/2009	98.9	100	76.5	84.9	65.1	67.3	65.1	69.4
681M/2009	75.0	80.6	72.2	79.5	65.8	68.4	65.1	68.4
684C/2009	98.9	100	76.5	84.9	65.1	67.3	65.1	69.4
686O/2009	93.2	92.4	72.9	81.7	64.4	64.2	60.6	66.3
PA3M/2008	93.5	94.6	73.6	81.7	63.7	65.2	62.0	67.3

Table 2. Comparisons of nucleotide and amino acid sequences of a RNA 2 fragment including the variable region of betanodaviruses detected in bivalve molluscs with reference betanodavirus genotypes (RGNNV: AY32487; BFNNV: EU826138; SJNNV: AB056572; TPNNV: EU236149; Thiery et al., 2012). Percentage of similarity of pairwise distances are shown.

1.5 DISCUSSION

This is the first study focusing on betanodavirus in bivalve molluscs. Twenty seven new betanodavirus strains were detected in bivalve molluscs of different species, European countries and year of collection and genetically characterized.

The sporadic presence of betanodaviruses in marine invertebrate has been previously detected (Gomez et al., 2008b; Ciulli et al., 2010; Gomez et al., 2010; Panzarin et al., 2012).

In our study, the finding of betanodavirus in bivalve molluscs was evidenced in samples collected for a long period from 2008 to 2015, from different Euopean countries and three species showing a consistent presence of this virus in these hosts. Particularly, clams collected in the North-eastern Italy in a time span of 7 years resulted constantly positive for betanodavirus. Moreover, a different prevalence was shown in bivalve mollusc species; particularly, clams seem to be more frequently contaminated than others species such as oyster and mussel. The presence of the virus in bivalve mollusc might be a natural consequence of their biology. Bivalve molluscs are obligate filter feeders and can accumulate particles including viruses from the surrounding water (Serratore et al., 2014). The fossorial behaviour of clams could favorite the virus-host contact and virus retention, compared to suspended farming methods used for oysters and mussels. However, also the geographical origin could have influenced the different prevalence evidenced among bivalve mollusc species in our study.

Phylogenetic analysis of both RNA 1 and RNA 2 fragments of betanodaviruses detected in bivalve molluscs showed a wide range of strains mainly belonging to RGNNV genotype, the most reported genotype in Europe.

However, the RNA 2 genetic analysis showed the presence of one atypical betanodavirus (681M/2009), placing definitively it outside of all genotypes officially recognized of the genus *Betanodavirus*. Particularly, the virus 681M/2009 seems to be more similar to RGNNV and BFNNV genotypes than to SJNNV and TPNNV genotypes, but it did not cluster with any of them. Pairwise percentage analysis confirms this result, with a nucleotide and amino acid sequence identity to each genotype lower than that evidenced between different genotypes.

Phylogenetic analysis of viruses detected in bivalve mollusc showed no correlation with their host species and geographical origin clustering together viruses detected in Italian clams, mussels and French oysters.

The comparison of the viruses detected in bivalve molluscs with several betanodaviruses isolated from finfish in Southern Europe (Panzarin et al., 2012) shows the circulation of similar viruses in finfish and in bivalve molluscs. In analogy to a previous study, several subgroups were identified in RNA 1 and RNA 2 phylogenetic trees inside RGNNV genotype. Most of these clusters include both

bivalve molluscs and finfish viruses with different geographic origin, year of isolation and host status (wild/farmed). Accordingly, the bivalve mollusc betanodaviruses did not cluster separately from finfish viruses, but rather they reflect the epidemiological patterns of betanodavirus circulating in finfish in Southern Europe.

Furthermore, some recent bivalve mollusc viruses clustered with finfish viruses detected several years ago (1996-2000) showing the persistent circulation of these viruses.

Previous study revealed the presence of a betanodavirus closely related to RGNNV in marine invertebrate (Japanese common squid *Todarodes pacificus*) and showed that it has high pathogenicity to finfish causing severe mortalities after i.m. challenge (Gomez et al., 2010). Similarly, the high genetic identy of viral strains detected in bivalve molluscs to those found in finfish highlights the inter-specific exchange of betanodaviruses.

1.6 CONCLUSIONS

The finding of this study show that betanodaviruses are widespread also in bivalve molluscs, maybe more than we could expect.

Phylogenetic analysis of these viruses shows that strains detected in mollusc bivalve and in finfish are very closely related and that betanodaviruses detected in bivalve molluscs in different European countries from 2008 to 2015 mimic the epidemiological patterns of betanodaviruses previously detected in finfish in Southern Europe from October 2000 to November 2009.

Moreover, the nucleotide and amino acid sequence analysis of the strain 681M/2009 show the existence of a new betanodavirus strain not belonging to any of the already known betanodavirus genotypes.

Such a massive and variegate presence of betanodaviruses in bivalve molluscs greatly stresses the risks of transmission previously feared for other invertebrates. Molluscs bivalve reared in the same area of farmed and wild finfish could act as a reservoir of virus. Furthermore, the marketing of alive bivalve mollusc and the relaying activity, allowed by the European regulation, can pose also a real risk of spreading betanodavirus between different geographical areas.

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CHAPTER 2

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

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2.1 ABSTRACT

Redspotted grouper nervous necrosis virus (RGNNV), genus *Betanodavirus*, family *Nodaviridae*, is the causative agent of Viral encephalopathy and retinopathy (VER), otherwise known as Viral nervous necrosis (VNN), and is a virus that could infect more than 70 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 the bivalve mollusc *Ruditapes philippinarum* and the RGNNV a culture-based method was optimised. The bioaccumulation of the pathogenic RGNNV by *R. philippinarum* and the potential shedding of viable RGNNV from RGNNV-exposed clams was evaluated through a culture-based method. *R. philippinarum* clearly accumulate viable RGNNV in the hepatopancreas tissue and are 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 at risk susceptible cohabiting cultured fish. The presence of RGNNV-contaminated molluscs could behave as RGNNV reservoirs and may modify the virus epidemiology.

2.2 INTRODUCTION

Redspotted grouper nervous necrosis virus (RGNNV), a virus of the genus *Betanodavirus*, family *Nodaviridae,* is responsible for Viral encephalopathy and retinopathy (VER), otherwise know as Viral nervous necrosis (VNN), a disease with nervous signs and mortality in more than 70 fish species worldwide (Doan et al. 2016). Betanodaviruses are small, icosahedral viruses that contain two segments of positive-sense single-stranded RNA. The RNA1 (3.1 kb) and the 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 coat protein gene, betanodaviruses are divided into four 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). Viral nervous necrosis virus (VNNV) is frequently isolated during outbreaks of VER in several farmed fish species, including European sea bass (*Dichentrarchus labrax*) in the Mediterranean Sea (Panzarin et al., 2012; Vendramin et al., 2013). Moreover, VNNV 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).

Currently, no successful therapies or commercial vaccines, apart from one in Japan (OIE 2013), 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., 2016).

Recent studies have also demonstrated that a certain population of apparently healthy wild marine fish carries 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).

It is well known that pathogenic agents may be spread via water masses, wild carriers, or vectors and restrictions do not fully ensure the control of disease spread by these routes (Mortensen, 2000; Mortensen et al., 2006). Water is both a dilution and a transport medium, and the fate of pathogenic agents shed into the water is dependent upon a series of factors, including dilution, inactivation by

UV light or other physical and chemical factors, particle bonding and uptake in filter-feeding organisms or particle-feeding plankton (Noble & Fuhrman 1997; Sinton et al., 2002; Wilhelm et al., 2003). Accordingly, aquatic organism interaction, both in the case of natural or artificial environments, such as integrated multitrophic aquaculture (IMTA), 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 it is shed back into the environment, or is inactivated by the molluscs (Skår & Mortesen 2007; Molloy et al., 2013).

The presence of VNNV 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).

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 aim of this study was to examine the bioaccumulation of a pathogenic RGNNV by Manila clam and to investigate the potential shedding of viable RGNNV from RGNNV-exposed clams through a culture-based method. The potential role of clams as an RGNNV reservoir and the potential risks posed by RGNNV-contaminated molluscs are pointed out in this study.

2.3 MATERIALS AND METHODS

2.3.1 Clam maintenance and RGNNV screening

Batches of market-size Manila clam (*Ruditapes philippinarum*), hereafter referred to as clams, were obtained from a commercial clam trader and were reared in an artificial recirculation system (RAS) (Adriatic Sea) supplied with natural seawater, collected from the Adriatic Sea and thermostated 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, aeration and thermostated at 15°C.

Prior to all trials, 30 clams from each batch were screened for presence of VNNV-RNA via an RT-PCR assay followed by a nested PCR performed according to methods previously described (Ciulli et al., 2006; Nishizawa et al., 1994).

2.3.2 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 cell/cm^2. Cells were allowed to attach and acclimate for 24 h at 25°C in order to achieve 80% confluence.

The previously characterised (Ciulli et al., 2006) It/351/Sb isolate of RGNNV was propagated in SSN-1 cells grown at 25°C in L-15 medium containing 2% FSB. When the cells demonstrated a 75% cytopathic effect (CPE), the cells and supernatant were centrifuged at 500 *g* for 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 Spearman-Karber method (Hierholzer & Killington 1996).

2.3.3 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* for 5 min and the supernatant was filtered through 0.20-μm-poresize 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* for 5 min. The supernatant was diluted 10-fold in L-15 with 2% FBS (Gibco).

Faecal matter samples were centrifuged at 3000 *g* for 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 five wells of a 96-well plate containing 24 hours-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 days. 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}.

2.3.4 Detection limit of $TCID_{50}$ endpoint dilution assay in RGNNV-inoculated clam hepatopancreas homogenates

Hepatopancreas from seven VNNV-RNA-negative clams were weighed, diluted 1:9 (wt/vol) with L-15 containing 2% FBS (Gibco) and homogenised before centrifuging at 3000 g for 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 six of the seven 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.

2.3.5 RGNNV clam exposure trial

In order to measure RGNNV uptake in clams, three independent exposure trials were performed. In each trial, 60 mussels were placed in 5 l plastic tanks containing 2 l of seawater thermostated at 15°C. An air-lift pump circulated the water and provided aeration. RGNNV suspension in L-15 cell culture medium was then added up to 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.

Hepatopancreas results were expressed as the mean ± standard deviation (SD) of positive samples obtained from the three trials. The samples of faecal matter and water were analysed in two repeats and the results were shown as the mean of the positive repeats ± SD.

2.3.6 Clam RGNNV shedding trials

The clam's ability to shed viable RGNNV in water through faecal matter was evaluated with two subsequent trials.

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

Trial 2. The shedding trial 2 was carried out in the same manner as the 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, Suffolk, UK) solution, followed by running tap water. Ten ml of water, faecal matter and triplicate clam samples were harvested for culture assays prior to each daily movement.

Hepatopancreas results of shedding trials were reported as the mean of positive samples ± SD. The samples of faecal matter and water were analysed in two repeats and the results were shown as the mean of the positive repeats ± SD.

2.3.7 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. The level for accepted statistical significance was $p < 0.05$. Positive data of culture assays, after testing for normality, were analysed by one-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). The level for accepted statistical significance was $p < 0.05$.

2.4 RESULTS

2.4.1 Clam maintenance and VNNV screening

The VNNV screening showed that all the batches involved in the trials were negative for VNNV-RNA presence. During all trials, no mortality was recorded in batches of clams used.

2.4.2 Detection limit of $TCID_{50}$ endpoint dilution assay 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 $TCID_{50}$ 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 of predicted value was associated with a decrease of the measured value ($R^2 = 0.96$, $F(1.4) = 91.64$, $y = 1.206x - 1.562$). 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 predicted titre of 1.7 log $TCID_{50}$ ml^{-1} and lower, no virus was detected by culture assays.

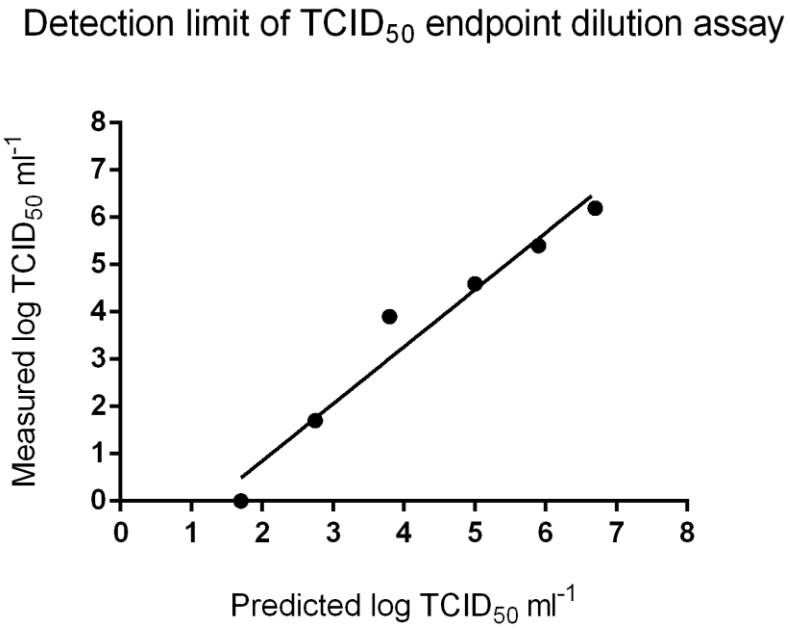

Fig. 1. Measured (•) and predicted (–) log $TCID_{50}$ ml^{-1} of RGNNV-inoculated clam hepatopancreas homogenates determined in SSN-1 cells.

2.4.3 RGNNV clam exposure trial

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

Eight of the nine 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 six of the nine 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).

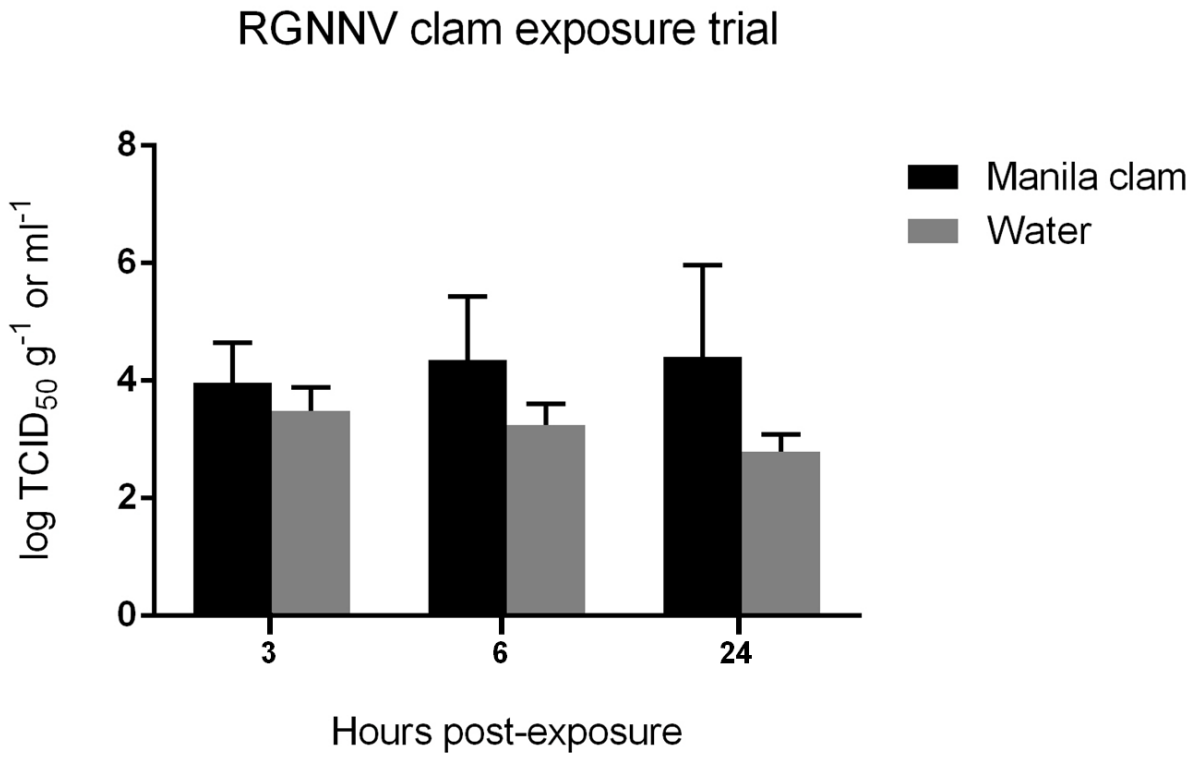

Fig. 2 Graph represents the log $TCID_{50}$ ml^{-1} or g^{-1} of RGNNV in clam hepatopancreas and water samples over time.

2.4.4 Clam RGNNV shedding trial

Trial 1. Viable RGNNV was isolated from all the clams sampled (Fig. 3). The RGNNV mean titre was $5.0 \pm 0.2 \log \mathrm{TCID}_{50}\, g^{-1}$; no statistical significances were shown 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}).

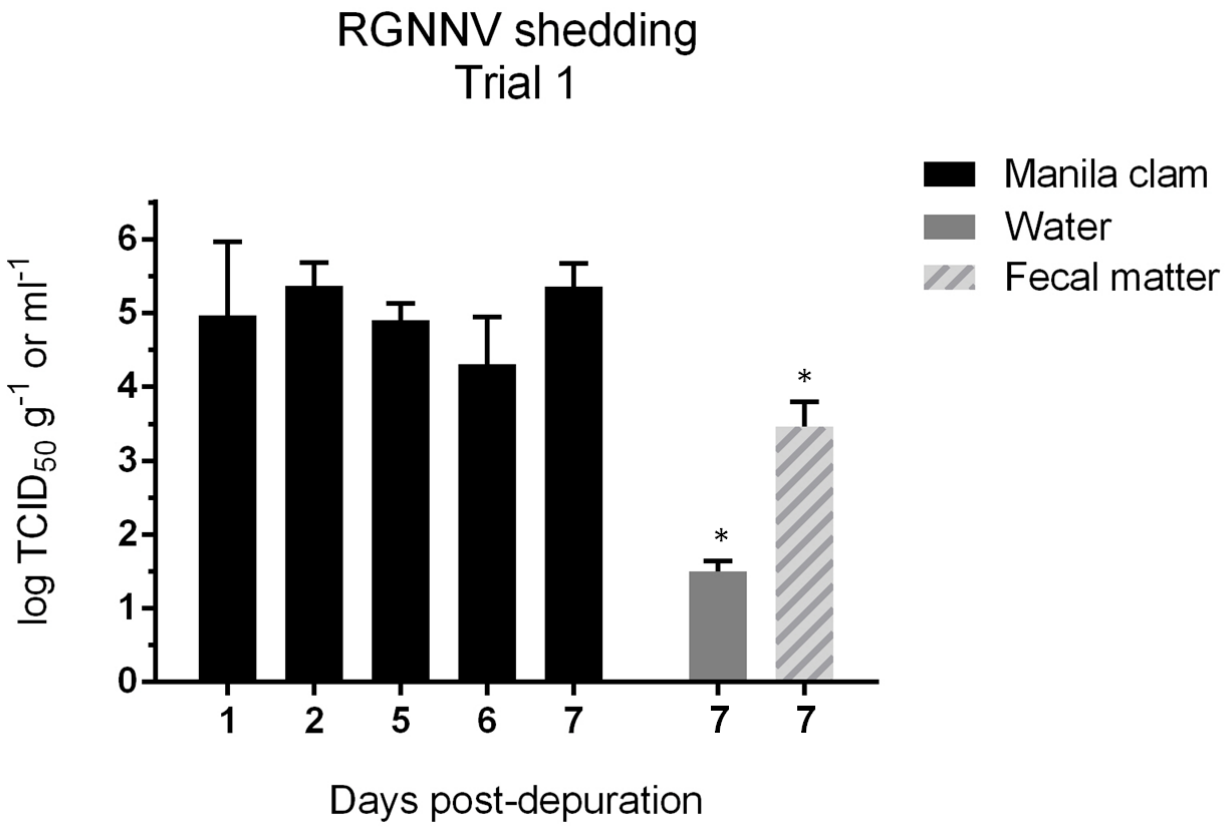

Fig. 3. Graph represents the log TCID_{50} ml^{-1} or g^{-1} of RGNNV in clam hepatopancreas , water and faecal matter samples over time. The asterisks indicate statistically significant different values from Manila clam values.

Trial 2. Viable RGNNV was isolated from all the 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. 4).

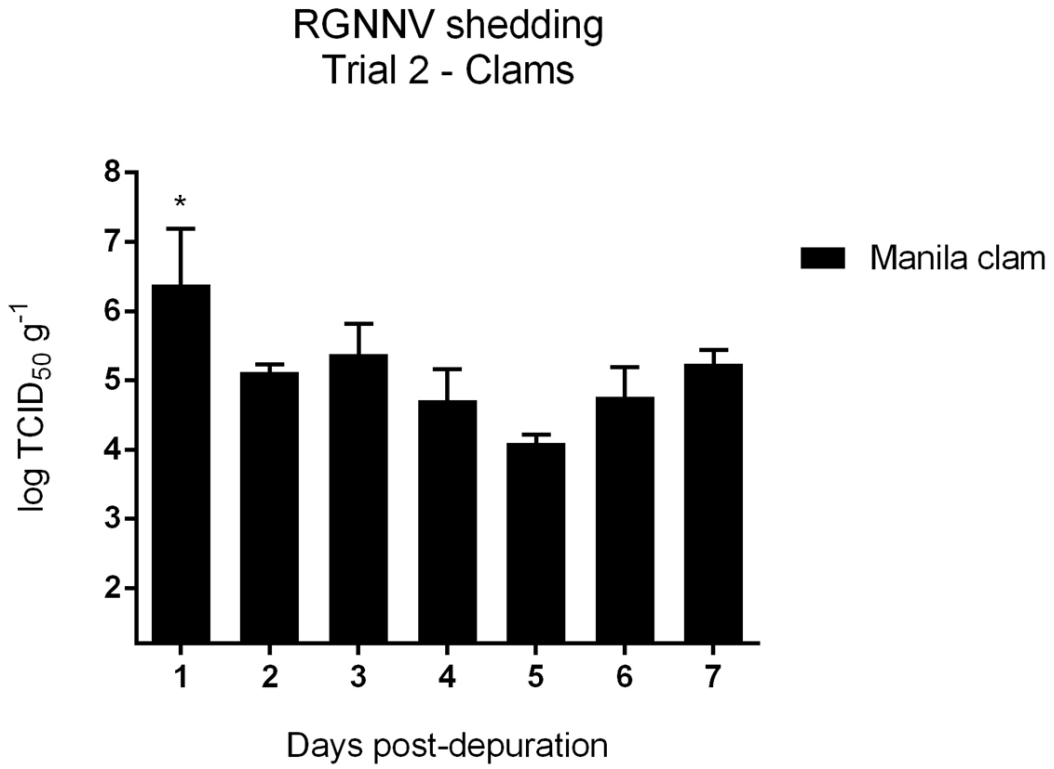

Fig. 4. Graph represents the log $TCID_{50}$ g^{-1} of RGNNV in clam hepatopancreas samples. The asterisk indicates a statistically significant different value from 2, 4, 5, 6 dpd samples.

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 one repeat of the water sample reported viable RGNNV. No statistically significant differences were revealed among virus titres detected at different time points (Fig. 5). In faecal matter, viable RGNNV was isolated from both repeats of all the samples with a mean titre of 3.9 ± 0.5 log $TCID_{50}$ g^{-1} except from one 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 statistical differences were shown among other time points (Fig. 6).

In water samples, the titrations showed statistically lower values than in the hepatopancreas tissues and then in the faecal matter samples at all tested time points ($p < 0.05$).

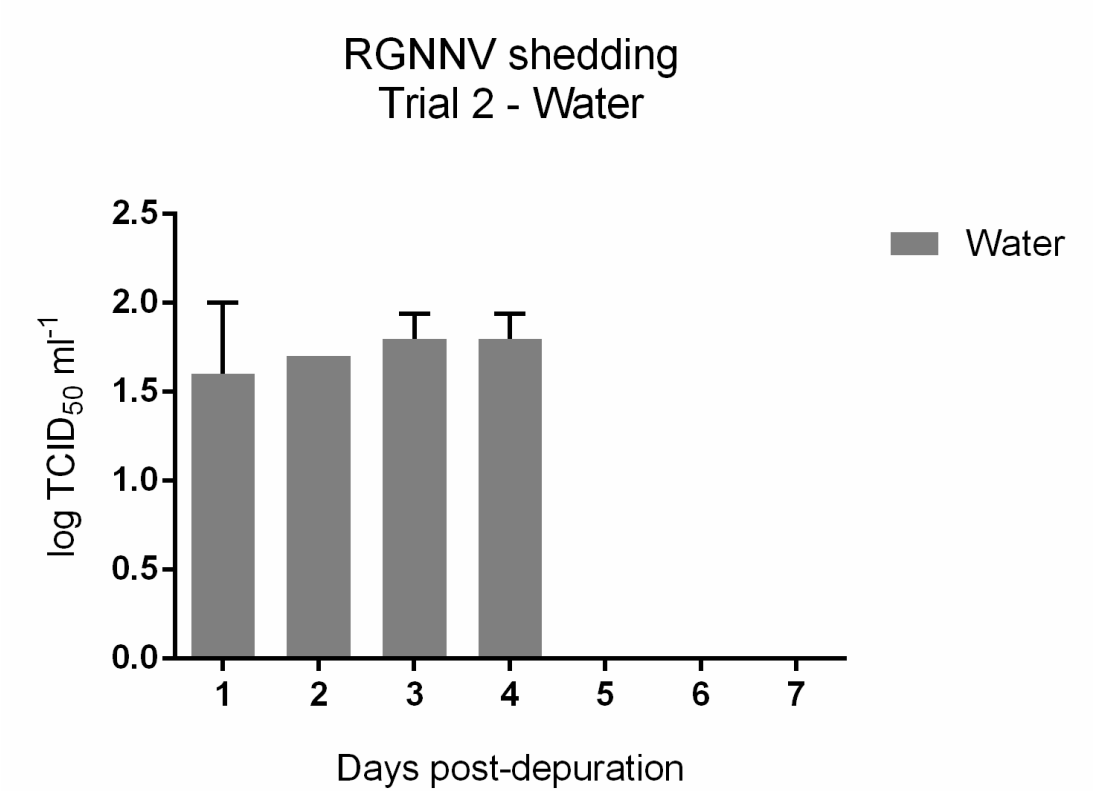

Fig. 5. Graph represents the log $TCID_{50}$ ml^{-1} of RGNNV in water samples over time.

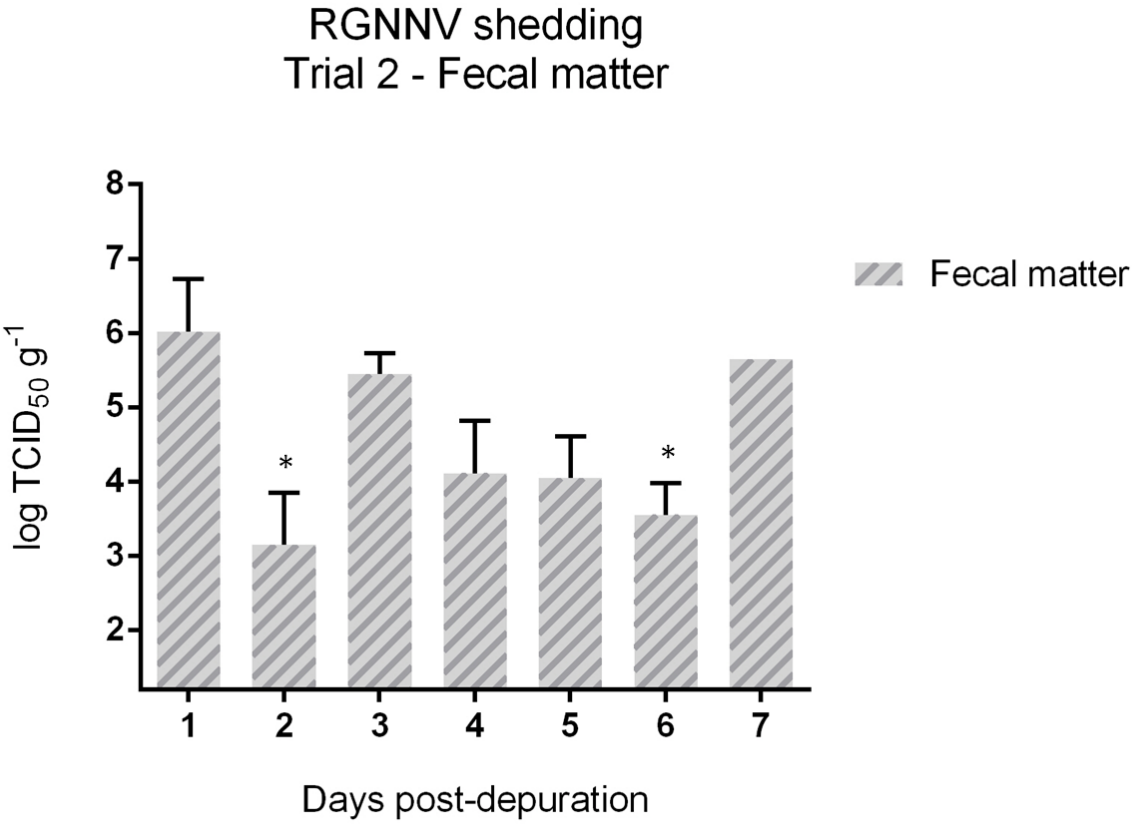

Fig. 6. Graph represents the log $TCID_{50}$ g^{-1} of RGNNV in faecal matter samples. The asterisks indicate statistically significant different values from 1 dpd sample.

2.5 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).

In order to understand the fate of RGNNV in virus-exposed clams, 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 was optimised. Through this culture assay, we were able to determine whether or not clams bioaccumulate viable RGNNV after experimental exposure to the virus, and to determine their proficiency to shed viable RGNNV particles into the surrounding environment.

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).

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). As a matter of fact, mussels (*Mytilus edulis*) are capable of bioaccumulating finfish viral pathogens, such as infectious salmon anaemia virus (ISAV) and infectious pancreatic necrosis virus (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 this study, Manila clam had clearly accumulated viable RGNNV in the hepatopancreas tissue. During the 24 h exposure trials, time did not show a statistically 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 was 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 mussel during a 120 h trial, showed that mussels significantly accumulate viable IPNV in their digestive gland tissues over time (Molloy et al., 2013). However, this study also showed that IPNV particles were not efficiently removed from the water column. 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 Hepatitis A (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 (Molloy et al., 2013; Bosch et al., 1995).

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 days post-depuration, respectively. Moreover, Trial 2 showed the amount of viable virus shed daily into the surrounding environment by RGNNV-exposed clams, and the persistence 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 (2013, 2014) and Skår & Mortensen (2007) seems to demonstrate that the inactivation of viruses is influenced by their morphology. In particular, nonenveloped 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.

Actually, 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 & Mortesen, 2007). Hence, it is difficult to predict whether wild or cultured clams near to farms of susceptible species might act as the cause 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*) (Gomez et al., 2008b, 2008c; Chi et al. 2003; 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 VNN in susceptible cultured fish.

2.6 CONCLUSIONS

Finally, the cell culture method set up in this study has allowed an 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 presents a serious risk for susceptible cohabitant fish species.

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. According to the results of this study, there is little doubt that the placing of contaminated molluscs into a fish farm, without proper control, could represent a serious risk for farmed fish.

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CHAPTER 3

Sea water disinfection by a peroxy-acid compound as a novel practice to complement and improve the microbial depuration of clams (*Ruditapes philippinarum*)

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3.1 ABSTRACT

A novel sea water disinfection process to complement and improve the microbial depuration of clams (*Ruditapes philippinarum*) was tested by employing a potassium peroxymonosulfate (MPS)-based product. A broad and multidisciplinary approach was used to achieve a quite complete pattern of the potential suitability of this innovative MPS-based disinfectant treatment to improve the microbiological quality of cultured Manila clam. The biocidal activity was evaluated against the *Vibrio* spp. population naturally associated with sea water and against viral nervous necrosis virus (VNNV), genus *Betanodavirus*, the most threatening among the viral pathogens of marine finfish.
The novel depuration process by sea water potassium MPS-based disinfection set up and tested in the present study (1000 ppm disinfectant concentration for three hours) clearly improves the microbiological quality of harvested clams with respect to their *Vibrio* spp. load. Furthermore, the proposed treatment greatly reduces the tested bacteria and viruses in the sea water column, being able to counteract possible microorganisms released from shellfish during the depuration process. The potential use of this treatment is strengthened by the absence of effects on clam health and welfare as well as of undesired side-effects and surfactant residues in the edible flesh. The whole of data suggests that the proposed treatment may efficiently reduce the risks related to the shellfish placing on the market, movement, depuration, relaying or consumption by humans. In practice, it

may potentially represent an innovative strategy to face, in a quite easy and feasible way, some still unresolved matter of concern in clam commercialization.

3.2 INTRODUCTION

Bivalve shellfish, hereafter referred to as shellfish, are important food sources for other organisms including humans. Most of the worldwide shellfish production (89.6%) comes from aquaculture, which plays a relevant role in ensuring food and good occupational opportunities to humans (FAO, 2013).

Shellfish are obligate filter feeders, gaining nourishment from microalgae, bacteria and organic particles in the surrounding water. In this way, they can also accumulate chemicals, marine biotoxins, bacteria and viruses that are pathogenic for humans and animals (Molloy et al., 2013; Serratore et al., 2014).

As stressed by the World Health Organization (WHO), the most effective and reliable approach to control the microbiological contamination of shellfish is to harvest them from areas with good water quality. Accordingly, the reduction of contamination through post-harvest processing procedures is known to be poorly effective, even if accepted as a practical option for production and trade of live shellfish on a large worldwide scale (Lees et al., 2010). Despite depuration is extensively practiced throughout the world, including Europe, North America, Asia and Australia, it is generally accepted that the results of this treatment are only adequate for shellfish with moderate levels of faecal contaminants, as it has very limited effectiveness on viruses and marine bacteria, such as *Vibrio* spp. (Lees et al., 2010; Serratore et al., 2014; Polo et al., 2014).

As an alternative to depuration, relaying has been proposed. This procedure consists of transferring contaminated shellfish to cleaner areas, so as to allow self-purification in the natural environment for long periods. However, this process, besides having effectiveness limitations (Oliveira et al., 2011), poses an additional risk of dissemination of autochthonous marine pathogens released by the shellfish after being accumulated by filtration in other geographical areas. This is the case of some fish pathogens such as bacteria of the genus *Vibrio* and naked RNA viruses, which were naturally found in shellfish (Mortensen et al., 1990; Ciulli et al., 2010; Pietrak et al., 2011**)**. A potential risk of viral transmission to other aquatic animals by virus releases by shellfish was reported for the infectious pancreatic necrosis virus (IPNV) (Molloy et al., 2013).

The recirculating aquaculture systems (RAS) are increasing employed facilities allowing the re-utilization of the waste-water, outflowing from the tanks containing the animals, by a continuous flux of collection, filtration, disinfection and redistribution. These systems only require the

replacement of the water lost by evaporation, shellfish transfer and plant cleaning (Serratore et al., 2014).

The results strongly depend on the plant efficiency to ensure a good water quality and to maintain the optimal physicochemical conditions to promote filtration by shellfish. Ultraviolet (UV) irradiation and/or ozonation can be used to treat and disinfect water before it returns to the tanks (Summerfelt et al., 2009) to avoid the gathering of pathogens and contamination among different batches.

In addition to the traditional disinfectant methods, peroxy acids with strong oxidizing effects are arising a great interest for disinfection in aquaculture, not only for their effectiveness, but also for the low environmental impact due to the poor toxicity of the by-products and minimal residues (Azanza 2004). However, high variability in pathogen susceptibilities to peroxy-based products was reported (Martin et al., 2013; Morin et al., 2015). Additionally, even if preliminary studies showed a potential *in vivo* application of the peroxy-based disinfectants, further studies are required to check their potential toxicity (Volpe et al., 2011; Straus et al., 2015). Low concentrations of peracetic acid were a tolerable stressor to carp, as shown by the cortisol measurement (Liu et al., 2015).

In the present work a novel sea water disinfection process was tested on Manila clam *(Ruditapes philippinarum)* by employing a potassium peroxymonosulfate (MPS)-based product. The objective was to obtain, through a broad and multidisciplinary approach, a quite complete pattern of the potential suitability of this innovative MPS-based disinfectant treatment to improve the microbiological quality of cultured Manila clam, in view of their safe commercialization. An increased microbiological quality of Manila clam puts on the market is of key importance since this allochthonous shellfish is now the first species in Italian aquaculture and its economic impact is great, being Italy the first producer in Europe (Sicuro et al., 2016)

First of all, it was deemed essential to assess the disinfectant ability to reduce the viral and bacterial load in sea water. The biocidal activity was evaluated against the *Vibrio* spp. population naturally associated with sea water and against viral nervous necrosis virus (VNNV), genus *Betanodavirus*, the most threatening among the viral pathogens of marine finfish (Doan et al., 2016).

On these bases, the ability of the disinfectant to reduce viral and bacterial load inside the Manila clam, in one word, its depuration efficiency, was investigated. At the same time, it was equally important to check the potential effects of the treatment on clam health and welfare as well as the absence of undesired side-effects and disinfectant residues in the edible flesh. Among the potential detrimental effects on seafood, the production of peroxidation products and the deterioration of organoleptic features were especially feared issues. Targeted parameters were tracked in parallel in

treated and untreated Manila clam to monitor health and welfare and verify the preservation of seafood quality and safety.

Some preliminary results were presented as posters to National Conferences (Ciulli et al., 2013; Passalacqua et al., 2014; Pagliarani et al., 2015).

3.3 MATERIALS AND METHODS

3.3.1 Disinfectant and experimental design

To implement the Manila clam microbial depuration, a new sea water disinfection system was set up and tested. The process included the use of a commercial disinfectant that, on the basis of the safety data sheet, contains pentapotassium bis(peroxymonosulphate) bis(sulphate) (40-55 %); sodium C10-13-alkylbenzenesulfonate (10-12 %); sodium dodecylbenzenesulfonate (7-10 %); sulphamidic acid (4-6 %); sodium toluenesulphonate (1-5 %); dipotassium peroxodisulphate (< 3 %); dipentene (< 0.25 %). The disinfectant efficacy in sea water was preliminarily tested against *Vibrio* spp. populations naturally associated to sea water and against a representative viral agent, VNNV, genus *Betanodavirus*, family *Nodaviridae*. This virus is widely distributed in the marine fauna (Doan et al., 2016), naturally bioaccumulated by Manila clam, highly resistant to chemical compounds and easily *in vitro* cultivated (Arimoto et al., 1996; Ciulli et al., 2010). Then, the biocidal activity was tested in an experimental depuration system, taking into consideration the effect of the disinfectant on Manila clam, other than on their microbial content, on some biochemical parameters, on cortisol level and on the histological pattern of selected clam tissues.

Due to the strong oxidation power of the disinfectant, which once dissolved in water generates H_2O_2, the biochemical analyses were focused on the oxidative damage to edible tissues of clams, which was a feared likelihood. Accordingly, the species is especially susceptive to lipid peroxidation promoted by environmental contaminants (Velez et al., 2015). The combination of methodologies applied was designed to identify both the occurrence of end-products formed by lipid peroxidation (Ayala et al., 2014) and changes in the volatile markers of the natural flavour of seafood, which can be used to signal possible alterations due to the treatment with the disinfectant before commercialization (Fratini et al., 2012).

Cortisol levels were investigated as a typical stress indicator. Accordingly, cortisol- like molecules and cortisol were described in haemocytes of several molluscan species (Ottaviani et al., 1998; Porte et al., 2006) including bivalve shellfish such as *Mytilus galloprovincialis.* Cortisol involvement in shellfish stress was recently investigated (Lagos et al., 2015).

Furthermore, treatment effects on clam tissues were assessed by histopathological investigations focusing on gills and digestive gland. Digestive cell lysosomes of shellfish, besides their role in intracellular digestion of food materials, constitute the main sites of toxic metal and organic pollutant sequestration and detoxification. In addition, neutral lipid alterations in the digestive gland of mussels are usually considered as environmental pollution biomarkers. Finally, lipofuscin deposition is also regarded as indicative of contaminant exposure, revealing a general response to pollution (Koukouzika et al., 2009).

For an accurate quantitation of potential residues of disinfectant in the clams the sodium dodecylbenzenesulfonate (LAS12) was chosen to overcome the problem of the high instability of the disinfectant main compound (pentapotassium bis(peroxymonosulphate) bis(sulphate)). Besides LAS12 was considered as representative molecule of all alkilbenzensulfonates of the product.

3.3.2 Bactericidal activity assay

The bactericidal activity was tested against the *Vibrio* spp. population naturally associated with sea water. For this assay, six 50L sea water tanks were set up to realize adequate mesocosms. Water was thermostatically controlled at 15±0.3°C, and each tank was equipped with a pump for internal recirculation and an aerator for oxygenation. Four concentrations of the disinfectant (1000, 100, 10, 1 ppm) were tested. All the assays were carried out in duplicate, devoting two tanks for each treatment (two treatments at a time); two tanks were used for the controls which were submitted to the same procedure except for the disinfectant addition. Water was sampled at 0, 1, 3 and 24 hours post-treatment (W-T0, W-T1, W-T3, W-T24). At each sampling time chemical-physical parameters (pH, dissolved oxygen, temperature, salinity) were measured. Bacteriological assays to detect and quantify *Vibrio* spp. load were conducted on selective medium TCBS (thiosulfate-citrate-bile salts-sucrose) Agar, Oxoid, NaCl 3%, providing with biochemical and functional tests to the genus confirmation (Hara-Kudo et al., 2001; Serratore et al., 2009).

3.3.3 Virucidal activity assay

The virucidal activity was specifically tested against the viral nervous necrosis virus (VNNV) of the genus *Betanodavirus*. The strain It/351/Sb of the VNNV, used for all the analyses, was previously isolated from naturally betanodavirus infected European sea bass (*Dicentrarchus labrax)* fry during an outbreak of Viral Nervous Necrosis (VNN) in the Adriatic Sea, Italy and genetically characterized by sequencing as a *Red spotted grouper nervous necrosis virus* (RGNNV; Genbank accession number: AY620367). To evaluate the virucidal activity of the disinfectant, an already

available CEN (Comité Européen de Normalisation) quantitative suspension test standard, BS EN 14675:2006, was modified by using the VNNV, RGNNV genotype, for a contact time of 1, 3 and 24 hours and a temperature of 15 °C (W-T1, W-T3, W-T24). Interfering substance was used as described for low level soiling in the standard (3 g/L bovine albumin solution).Three concentrations (1000, 500, 100 ppm) of the disinfectant were tested. Test solutions of the disinfectant were prepared in sterilized sea water at 1.25× the test concentrations. Briefly, the virus was propagated and titrated in striped snakehead (SSN-1, *Channa striatus*) cells at 25 °C. The 50% tissue culture infectious dose ($TCID_{50}$) was calculated according to the method of Spearman-Kärber (Hierholzer & Killington, 1996). One part of interfering substance, for low-level soiling conditions, was mixed with 1 part of a suspension of $10^{6.75}$ $TCID_{50}$ mL^{-1} VNNV and equilibrated to 4±1.0 °C in a water bath. Eight parts of the disinfectant were added to the mixture and briefly vortexed. The test mixture was left for the set contact time of 24 hours. At each sampling times the text mixture was vortexed and 20 µL immediately removed and 10-fold diluted in cell culture maintenance medium Leibovitz-15 medium (Gibco, Life Tech-nologies, Paisley, UK) supplemented with 10% Fetal Bovine Serum (FBS; Gibco, Life Technologies), 1% L-glutamine (Gibco, Life Technologies) and 1% antibiotic-antimycotic solution (Gibco, Life Technologies). The diluted test mixture was added to 5 wells of a 96 well microtitre plate containing a monolayer of 80% confluent SSN-1 cells. The titrations were incubated at 25 °C for 7 days, after which they were examined for cytopathic effect (CPE) using an inverted microscope and the titre expressed as $TCID_{50}$ mL^{-1} calculated according to the method of Spearman-Kärber (Hierholzer & Killington, 1996). The assays were carried out in triplicate.

3.3.4 Depuration assay

All depuration assays were conducted using cultured Manila clam (*R. philippinarum*), a species which is not included within the scope of the Directive 2010/63/EU on animal used for scientific purpose, therefore all the experimental trials did not require the approval of the Ethical Committee.
The assays were carried out in four, 50L sea water mesocosms. The water was thermostatically controlled at 15±0.3°C. Each tank was equipped with a pump for internal recirculation and an aerator for oxygenation. Prior to the experimental depuration assays, clams were acclimated at 15°C for three days and maintained under natural photoperiod. During acclimation, clams were sampled and tested for the presence of the following viral and bacterial natural contaminations: human norovirus genogroups GI and GII (NoV GI, GII), hepatitis A virus (HAV), viral nervous necrosis virus (VNNV), *Vibrio* spp., using previously described methods (Le Guyader et al., 1994; Vinjé et al., 1996; Green et al., 1998; Hara-Kudo et al., 2001; Boxman et al., 2006; Ciulli et al., 2007; Serratore et al., 2009).

Three concentrations (1000, 500, 100 ppm) of the disinfectant were tested by diluting the commercially available disinfectant solution in sea water up to attain the concentrations to be tested. All the assays were conducted in duplicate, devoting two tanks for the treatment (one treatment at a time) and two tanks for untreated controls.

The clams were sampled after 0, 1, 3 and 24 h treatment (MC-T0, MC-T1, MC-T3, MC-T24) to detect and quantify *Vibrio* spp. in flesh and intravalvular liquid. At each sampling time clam mortality was checked and the water chemical-physical parameters (pH, dissolved oxygen, temperature, salinity) were measured.

On the basis of the results of these preliminary assays, the most effective concentration of 1000 ppm disinfectant and the best contact time of 3 hours were selected for the subsequent depuration assays. The treatment of 1000 ppm disinfectant for three hours was repeated four times to point out, through the evaluation of biochemical, endocrinological, histomorphological and chemical parameters, season-dependent responses. So, depuration assays were carried out under the same maintenance conditions in July, October, November and December to identify possible differences in the response to the disinfectant due to the environmental source and/or the physiological status of clams. Accordingly, seasonal variability was reported for several biomarkers and oxidative stress responses in Manila clam and other shellfish species (Bocchetti et al., 2008). In each trial, at the experiment start (MC-TO) and after 3 h treatment from each of the two tanks containing 1000 ppm disinfectant (MC-T3 1000 ppm) or maintained under the same conditions in disinfectant-free seawater (MC-T3 CTRL) clams were collected for all the analyses and differently stored according to their use. In detail, sampled clams were immediately frozen and maintained at -80° (biochemical analyses) or - 20°C (endocrinological and chemical analyses), dissected and fixed in 10% buffered formalin and processed for routine histology. In October and December trials, after the treatment (1000 ppm for 3 hours) clams, to be collected for histopathological investigations, were maintained in untreated sea water by 1 day, to verify possible long-term alterations due to treatment.

During each trial, clams after 0, 1, 3 and 24 h treatment from each of the two tanks containing 1000 ppm disinfectant (MC-T0 1000ppm; MC-T3 1000ppm ; MC-T24 1000 ppm) or maintained under the same conditions in disinfectant-free seawater (MC-T0 CTRL; MC-T3 CTRL; MC-T24 CTRL) were collected and immediately processed for microbiological analyses.

Furthermore, water samples were randomly collected from each tank at 0, 3 and 24 h treatment (W-T0, W-T3, W-T24).

Since no natural viral contaminations (screening for NoV GI, GII, HAV, VNNV) were detected in the Manila clam batches employed, the depuration assay was focused on *Vibrio* spp. Bacteriological assays to detect and quantify *Vibrio* spp. load were conducted on selective medium TCBS Agar

NaCl 3% providing with biochemical and functional tests to the genus confirmation (Hara-Kudo et al., 2001; Serratore et al., 2009).

3.3.5 Artificial contamination with VNNV, *Betanodavirus* and specific depuration assay

A contamination trial was set up, placing the clams in a tank containing 2 L of seawater, previously contaminated with 10^5 $TCID_{50}$ mL^{-1} of VNNV, strain it/351/Sb. An uncontaminated control was set up by immerging clams in a clean sea water tank. The clams were allowed to filter and concentrate the seeded virus for 24 hours, then they were removed from the tank, rinsed under running water and immersed in a clean sea water tank for 24 hours. These artificially contaminated clams were subjected to the previously described depuration assay (Section 2.4). The assay was conducted with two repeats, namely contaminated clams were divided in four tanks, devoting two tanks for the treatment (1000 ppm) and two tanks for controls in disinfectant-free water. Three clams were randomly collected at 0, 1, 3, 24 hours post treatment to detect the virus presence in the digestive gland (MC-T0, MC-T1, MC-T3, MC-T24). VNNV was detected and quantified by virus isolation on SSN-1 cell culture as previously described (Section 2.3).

3.3.6 Biochemical analyses

Immediately after thawing, 50 clams from each sampling (MC-TO, MC-T3 CTRL and MC-T3 1000 ppm) were opened and divided into two pools of 25 clams each, randomly selected. The soft tissues were carefully removed from the shell by a scalpel, rinsed in ice-cold medium (0.25M sucrose, 5mM tris(hydroxymethyl)-aminomethane, 5mM ethylenediammine tetra-acetic acid, pH 7.4), gently dried on blotting paper and weighted. All the subsequent analyses were carried out on each pool. The occurrence of oxidation products in edible tissues was checked by two different approaches which complement each other. Thiobarbituric acid reactive substances (TBARs), which can be formed as by-products of lipid peroxidation, were evaluated by a colorimetric assay (Banni et al., 2009) as equivalents of malondialdehyde (MDA).

The profile of volatile organic compounds (VOC), which provides qualitative information on the pattern of compounds in the soft tissue and is related to the organoleptic features of seafood, was obtained by solid-phase microextraction (SPME) coupled to capillary-column gas-chromatography (Fratini et al., 2012). The experimental conditions, optimized for the VOC extraction from clams, were as follows. Briefly, an 85 μm CAR/PDMS fibre was exposed to the head space of 5.0 mL saline extract of edible tissue, placed in a 10 mL crimp cap vial and subjected to magnetic stirring for 30 min at 50°C. Target analytes on the loaded fibres were thermally desorbed for 10 min at

260°C directly in the GC injector port used in splitless mode. Both absorption and desorption phases were controlled by the Varian CP-8200 autosampler. VOC were analysed on a Varian 3380 gas-liquid chromatograph equipped with a fused silica capillary column Equity Supelco (30mX0.25 mm i.d. and film thickness 0.25 μm) and flame-ionization detector (FID). The chromatographic conditions were: H_2 as carrier gas (2.0 mL min^{-1}); temperature programme: 35°C held for 5 min, 8°C min^{-1} to 75°C, 40°C min^{-1} to 200°C and final isotherm at 200°C for 5 min; FID held at 275°C.

3.3.7 Cortisol measurement

Cortisol was extracted following the method of Lagos et al. (2015), partially modified. Immediately after thawing, clams from each sampling (MC-TO, MC-T3 CTRL and MC-T3 1000 ppm) were opened, the digestive glands were collected, weighed to a final net weight 4 g for each sample and homogenized by means of an Ultra-turrax T25 for 3 min. Homogenates were extracted with diethyl ether (15 mL) with continuous gentle agitation on the rotor for 1 h. After centrifugation (2000 x *g* for 30 min), 13 mL of the ether phase was collected, transferred into a glass tube and evaporated to dryness under an air-stream suction hood. The dried extracts were dissolved in buffer and analysed using a RIA method (Tamanini et al., 1983). To validate RIA method, cortisol parallelism and intra-assay precision tests were performed. To determine the parallelism between cortisol standard and endogenous hormone in tissue, a sample containing high concentration of cortisol was serially diluted (1:1-1:8) with RIA buffer. Parallelism was assessed between these serial dilutions and cortisol standards (ranging from 7.81 to 1000 pg/100 μl tube vial, prepared in buffer). The intra-assay coefficient of variation was 9.7%. Cross reactions of various steroids with antiserum raised against cortisol were as follows: cortisol 100%, corticosterone 9.5%, cortisone 5.3%, 11a-deoxycortisol 5%, prednisolone 4.6%, 20a-dihydrocortisone 0.4%, progesterone and testosterone <0.001%. The sensitivity of the assay was 2.64 pg/tube and was defined as the dose of hormone at 90% binding. The results are given as pg/g tissue.

3.3.8 Histological examination

Soft tissues of clams were removed from the shells, and fixed for 24 hours in 10% neutral buffered formalin, routinely processed and embedded in paraffin wax. Sections (4 μm) were stained with haematoxylin and eosin (HE). The histological examination on HE focused on the gills and digestive gland to detect the presence or absence of cell changes (degenerations with water / lipid accumulation, necrosis or apoptosis). When on HE slide a brown pigment was present, sections

were stained with Ziehl-Neelsen to confirm the presence of lipofuscin (Gòmez-Mendikute et al., 2005; Koukouzika et al., 2009).

3.3.9 Sodium dodecylbenzenesulfonate (LAS12) quantification

For the quantification of sodium dodecylbenzenesulfonate (LAS12) in Manila clam tissues ultra-performance liquid chromatography coupled to triple-quadrupole mass spectrometry (UPLC-MS/MS) technology was used. The equipment employed consisted of a Waters Acquity UPLC® binary pump, coupled with a Waters Quattro Premier XE™ triple quadrupole mass spectrometer equipped with an ESCi™ Multi-Mode Ionization Source (Waters Corporation, Milford MA, USA). Mass spectrometer operated in negative electrospray ionization (ESI^-) mode and analysis were performed in MRM (multiple reaction monitoring) mode, following two specific transitions for the target analyte: 325.4 > 119.1 and 325.4 > 183.1. The chromatographic separation was achieved on a Waters Acquity BEH SHIELD RP18 UPLC® column (Waters Corporation, Milford MA, USA). The chromatographic conditions consisted in an isocratic run (Rico-Rico, Drogea, Widmera, & Hermensa, 2009) of 5 minutes at a constant flow of 0.4 mL/min; the mobile phase was 10:90 (v:v) of A:B, where A is 10 mM ammonium acetate aqueous buffer, and B is methanol. Data were acquired and processed using a Waters MassLynx™ 4.1 software (Waters Corporation, Milford MA, USA).

Clam tissue was processed through a simple liquid-liquid extraction procedure: homogenised clam tissue was twice extracted with methanol and ultrasonication, then the supernatant, obtained by centrifugation, was cleaned by means of two purification-steps with hexane. The extract was successively dehydrated with the addition of anhydrous sodium sulphate, concentrated under gentle stream of nitrogen, and re-suspended with the chromatographic eluent solution. Finally, the samples were filtered directly in polypropylene vials through nylon syringe-driven filters.

3.3.10 Statistical analysis

All data are expressed as the mean of repeats ± standard deviation (SD) and they were analysed by two-way ANOVA followed by Bonferroni or Tukey's post hoc tests (Prism version 4.0 software, GraphPad Software, San Diego, USA; SigmaStat 2.0, Sigma, USA). The level for accepted statistical significance was $p \leq 0.05$.

3.4 RESULTS

3.4.1 Bactericidal activity

The monitoring of chemical-physical parameters in the mesocosms set up for the disinfection treatment, showed that dissolved oxygen, temperature and salinity were not affected (Fig. 1A, B, C), unlike pH, by the different disinfectant concentrations. In detail, pH greatly decreased at 1000 ppm (ΔpH=3.40), slightly decreased at 100 ppm (ΔpH=0.65), whereas it remained constant at 10 and 1 ppm disinfectant (Fig. 1 D).

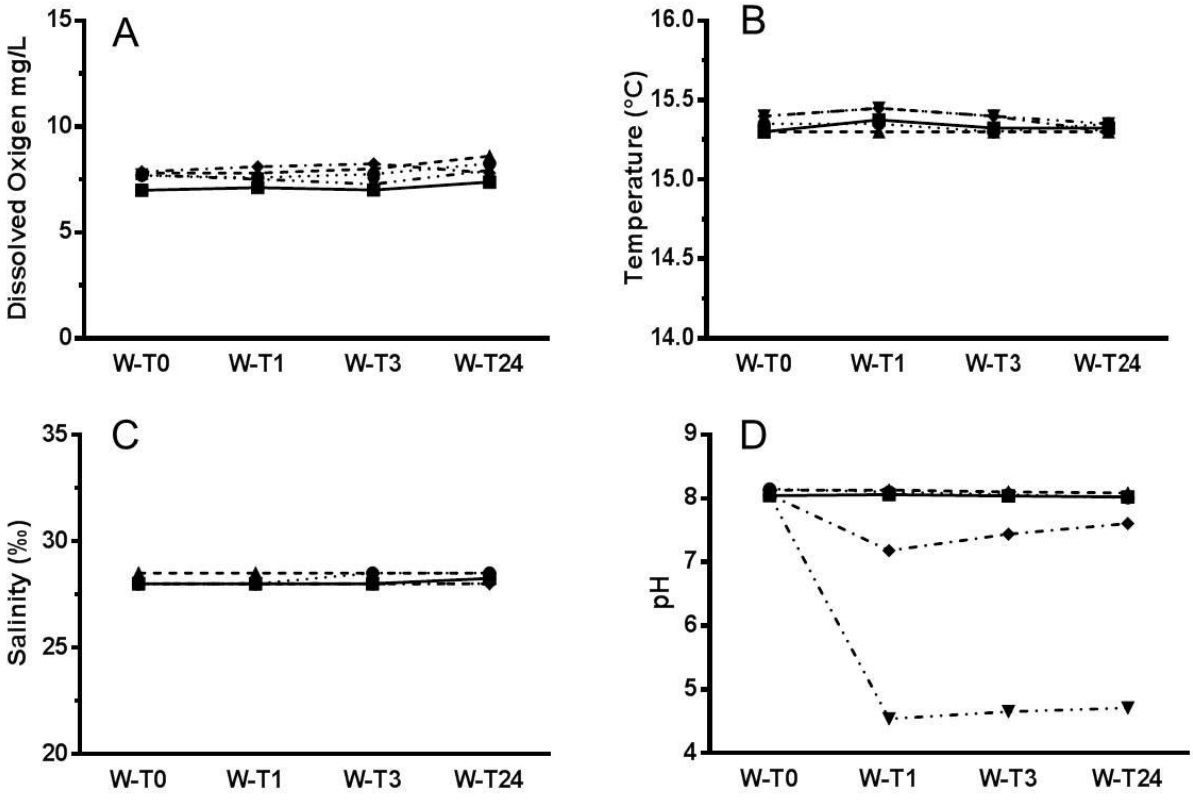

Fig. 1. Graphs show the evolution of the chemical-physical parameters in mesocosms set up for the bactericidal activity assays after the treatment with the disinfectant at different concentrations (CTRL, 1 ppm, 10 ppm, 100 ppm, 1000 ppm) and time points (W-T0, W-T1, W-T3, W-T24). A) Dissolved oxygen; B) temperature; C) salinity; D) pH. Data are presented as mean ± SD.

Sea water analyses showed a natural *Vibrio* spp. load of 2.8±0,1 $\log_{10}$ CFU mL^{-1} (W-T0). *Vibrio* spp. load was constant throughout the bactericidal activity assay in control tanks (Fig. 2).

On the other hand, *Vibrio* spp. associated to sea water after all treatment (1, 10, 100 and 1000 ppm) resulted rapidly reduced to statistically lower level than those of untreated controls (W-T1; $p<0.05$; Fig. 2). At 10, 100 and 1000 ppm disinfectant, *Vibrio* spp. could not be isolated from treated sea water samples at all the tested time points (W-T1, W-T3, W-T24). At 1 ppm, *Vibrio* spp., were isolated from sea water samples collected at 1, 3 and 24 hours post treatment, but with statistically lower bacterial loads than those of the untreated controls ($\Delta=2.25$ $\log_{10}$ CFU mL^{-1}; $p<0.05$; Fig. 2).

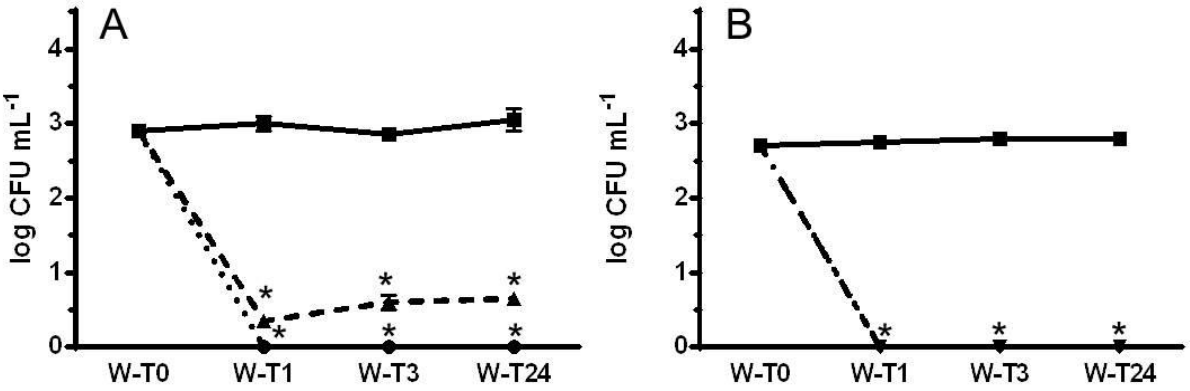

Fig. 2. Graphs show *Vibrio* spp. load in sea water samples collected during the bactericidal activity assays after the treatment with the disinfectant at different concentrations ■ CTRL, (A) ▲ 1 ppm, ● 10 ppm, (B) ◆ 100 ppm ▼ 1000 ppm and time points (W-T0, W-T1, W-T3, W-T24). Data are presented as mean ± SD. Asterisks (*) show statistically significant differences ($p < 0.05$) of *Vibrio* spp. load between treated and untreated samples collected at the same time point.

3.4.2 Virucidal activity

The treatment at 1000 ppm disinfectant reduced rapidly VNNV load to level statistically lower than those of untreated controls (W-T1 CTRL *vs* WT-1 1000 ppm, $p<0.05$). Accordingly, one hour after the treatment the viral load was about 3 $\log_{10}$ $TCID_{50}$ mL^{-1} lower than control (Fig. 3 A). Viral load in treated water was consistent with values statistically lower than those of controls up to 24 hours at each tested time points (W-T1, W-T3, W-T24; $p<0.05$; Fig. 3 A). After the 500 ppm treatment, the viral load decreased up to attain statistically lower values than that of the control after three hours (W-T3, $p<0.05$; Fig. 3 B). The 100 ppm disinfectant treatment did not cause a significant reduction of the viral load at any tested time points (Fig. 3 C).

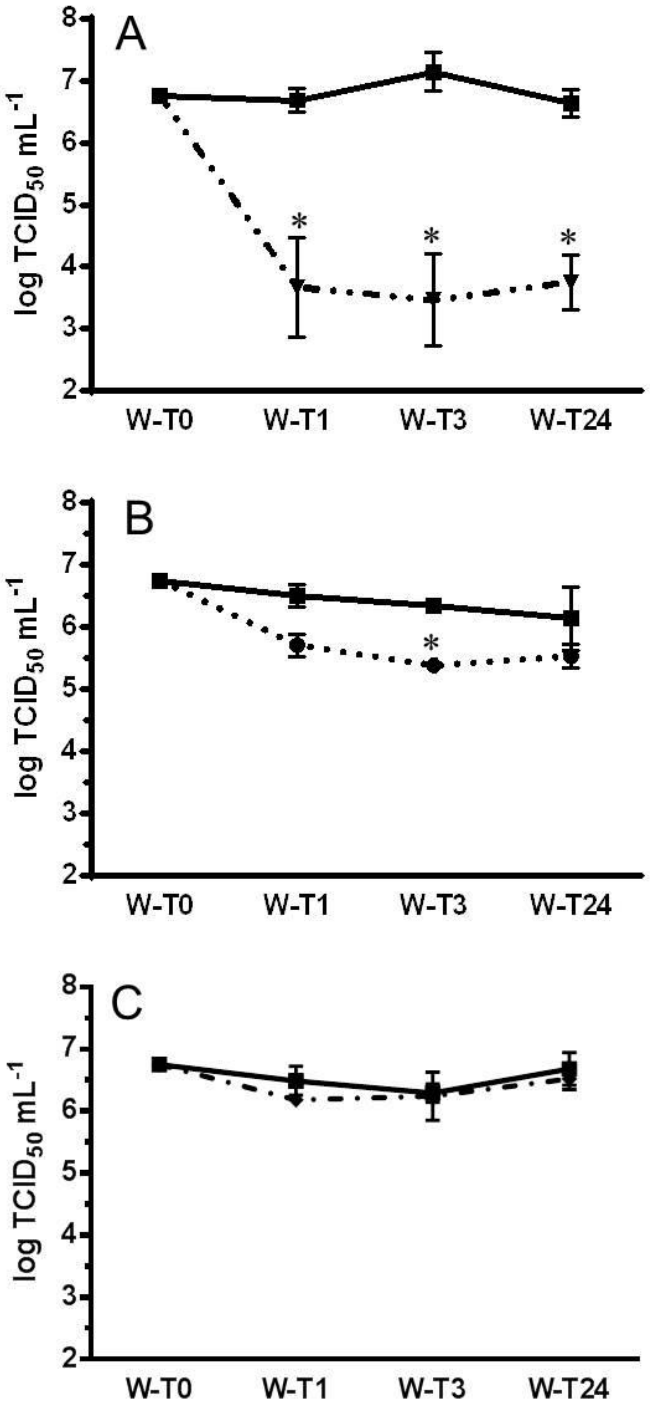

Fig. 3. Graphs show viral loads detected in sea water samples collected during the virucidal assays after the treatment with the disinfectant at different concentrations ▪ CTRL, (A) ▲ 100 ppm, (B) ● 500 ppm, (C) ▼ 1000 ppm and time points (W-T0, W-T1, W-T3, W-T24). Data are presented as mean ± SD. Asterisks (*) show statistically significant differences ($p < 0.05$) of VNNV load between treated and untreated samples collected at the same time point.

3.4.3 Depuration assay

The monitoring of chemical-physical parameters (pH, dissolved oxygen, temperature, salinity) in mesocosms during the depuration assay showed results similar to those obtained during the bactericidal activity assay (data not shown).

Clams analysed at the origin (MC-T0) showed *Vibrio* spp. loads ranging from 3.48 $\log_{10}$ CFU g^{-1} to 5.75 $\log_{10}$ CFU g^{-1} (Fig. 4 A, B, C) reflecting the normal *Vibrio* spp. loads naturally associated to flesh and intravalvular liquid (Serratore et al., 1999; Milandri et al., 2000; Serratore et al., 2009; Passalacqua et al., 2016). Both the 100 and 500 ppm disinfectant treatments did not result into any reduction of *Vibrio* spp. load associated to flesh and intravalvular liquid (Fig. 4 A, B). On the other hand, the 1000 ppm disinfectant treatment (five repeats) showed a different effect at the different sampling times. The *Vibrio* spp. load showed a statistically significant reduction after three hours of treatment ($p<0.05$; Fig. 4 C), whereas *Vibrio* spp. loads were overlapping for treated and untreated groups after 24 hours.

Furthermore, the monitoring of *Vibrio* spp. associated to sea water during the depuration assay showed performances overlapping those reached in the bactericidal activity assay. A reduction of about 3 $\log_{10}$ CFU mL^{-1} of the bacterial load with respect to the control was obtained in the water of the treated group three hours post treatment ($p<0.05$; Fig. 4 D). Particularly, *Vibrio* spp. was not isolated from sea water collected neither three nor 24 hours post 1000 ppm disinfectant treatment.

During the depuration assay no statistically significant differences in mortality arose among all treated and untreated clams.

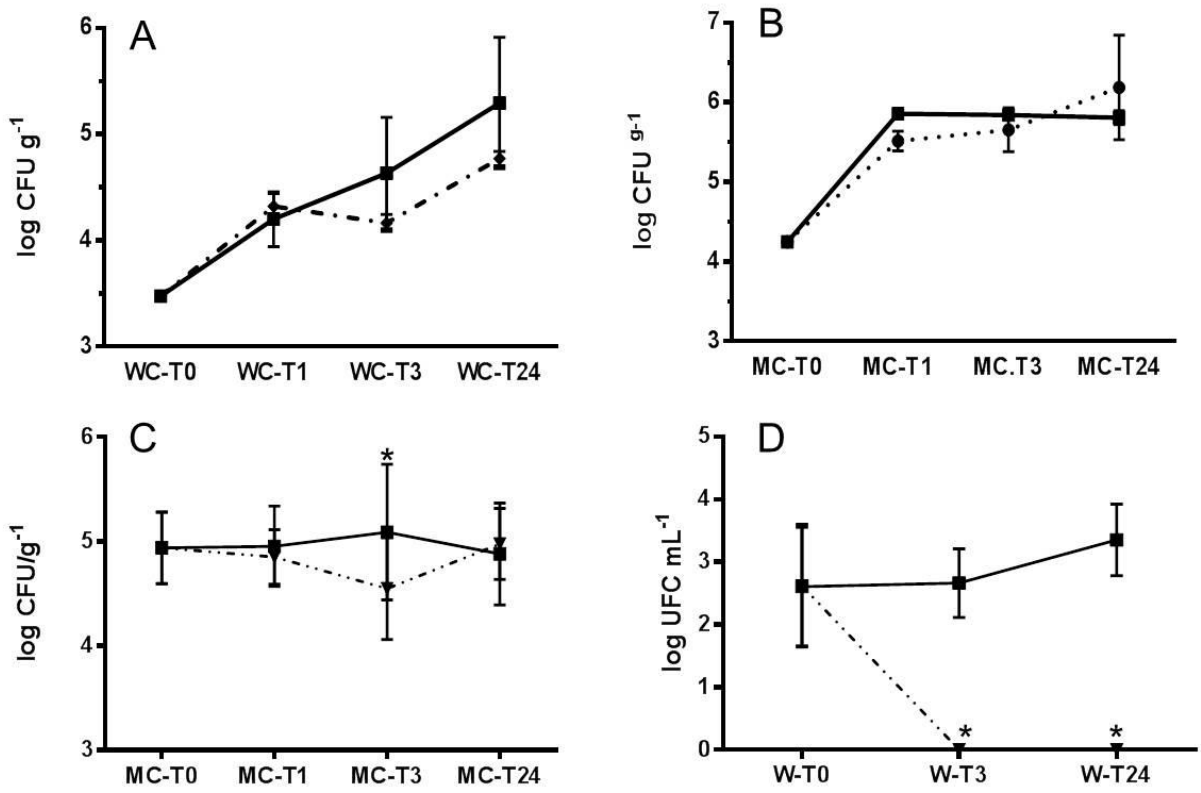

Fig. 4. Graphs show *Vibrio* spp. load in clams (MC) and water (W) collected during the depuration assays after the treatment with the disinfectant at different concentrations CTRL, (A) 100 ppm, (B) 500 ppm, (C, D) 1000 ppm and time points (T0, T1, T3, T24). Data are presented as mean ± SD. Asterisks (*) show statistically significant differences ($p < 0.05$) of *Vibrio* spp. load between treated and untreated samples collected at the same time point.

3.4.4 Artificial contamination with VNNV, *Betanodavirus* and specific depuration assay

The clams were successfully contaminated with VNNV by the immersion trial. VNNV was isolated from contaminated clams after 24hours immersion in contaminated water and subsequent 24 hour in clean water showing their ability to retain the virus. No mortality was observed neither in contaminated clams nor in the uncontaminated control. Contaminated clams showed a VNNV titre 4.6±1.6 $\log_{10}$ $TCID_{50}$ g^{-1} (MC-T0) at the starting time. No statistically significant VNNV titre reduction was observed after 1000 ppm disinfectant treatment at any of the tested time points (Fig. 5).

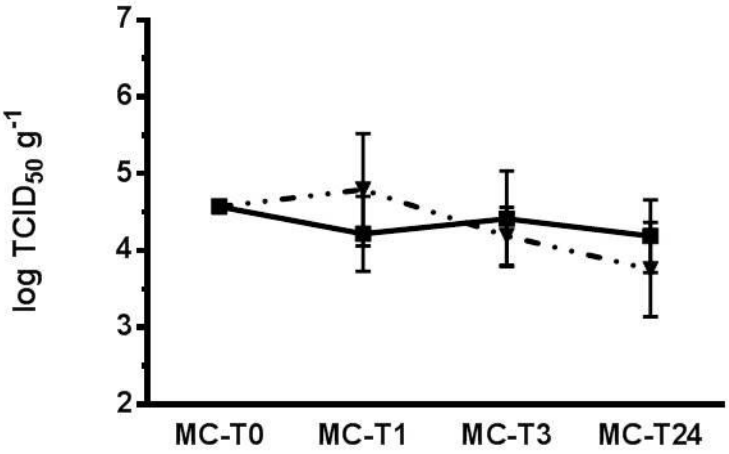

Fig. 5. Graph shows VNNV load in clams collected and time points (MC-T0, MC-T1, MC-T3, MC-T24) during the depuration assay after the treatment with the disinfectant (-■- CTRL, -▼- 1000 ppm). Data are presented as mean ± SD.

3.4.5 Biochemical analyses

The selected disinfectant treatment (1000 ppm for 3 hours) apparently did not affect the biochemical parameters considered. Irrespective of the season, in all the 1000 ppm exposure trials, the level of TBARs, evaluated as MDA equivalents, was not significantly different in disinfectant-treated (MC-T3 1000 ppm) and untreated (MC-T3 CTRL) clams (Fig. 6).

On the other hand, a clear effect of the sampling was pointed out. In the trials of October, November and December the MDA levels were significantly higher than in the trial of July, irrespective of the exposure to the disinfectant.

Moreover, as shown in Fig. 7, which illustrates a representative pattern of VOC in MC-TO, MC-T3 CTRL and MC-T3 1000 ppm clams, very similar and overlapping VOC spectra within each trial were obtained. The substantially overlapping VOC pattern in MC-TO, MC-T3 and MC-T3 1000 ppm was observed in all the trials (July, October, November and December) (data not shown).

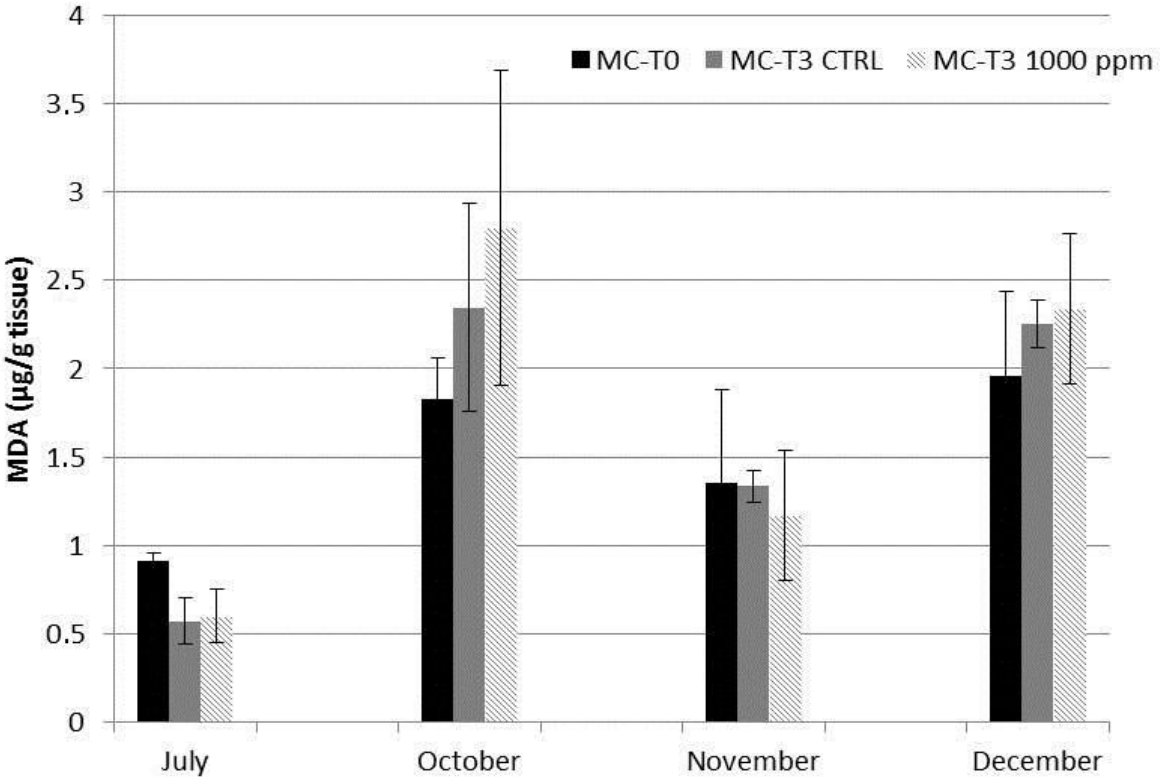

Fig. 6. MDA levels in edible tissues of MCT-O (■), MC-T3 CTRL (■) and MC-T3 1000 ppm (▨) clams.

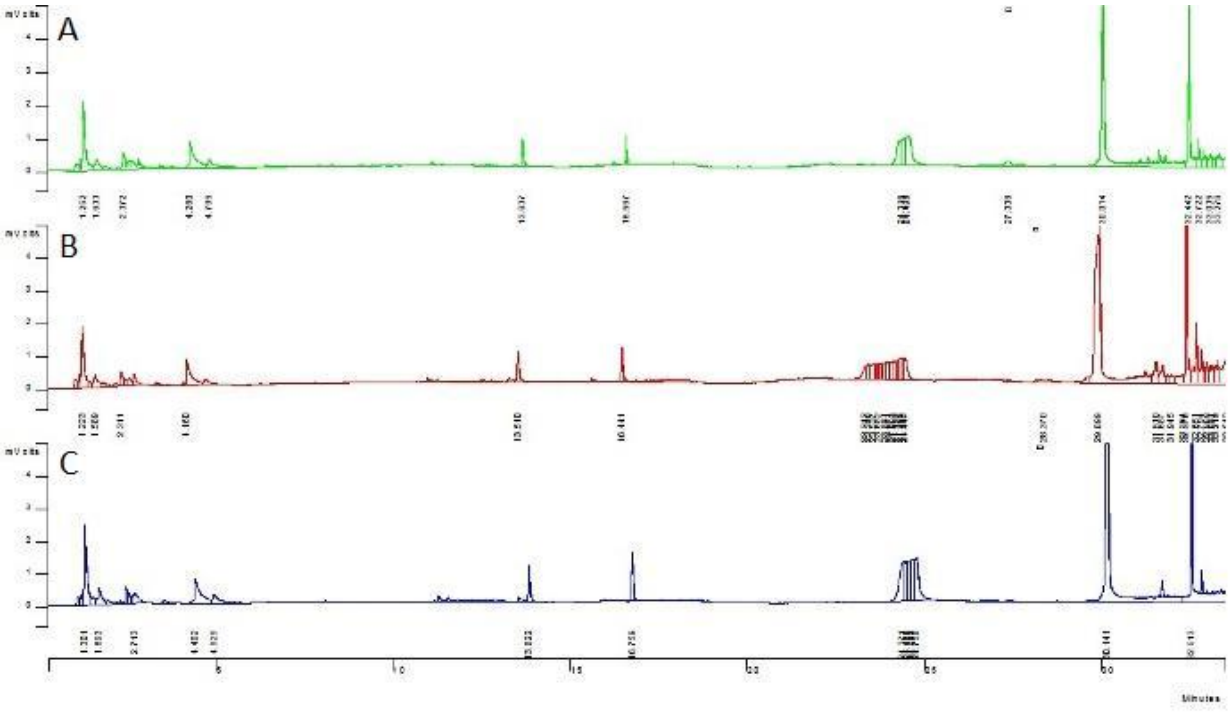

Fig. 7. Representative VOC patterns in MC-TO (A), MC-T3 CTRL (B) and MC-T3 1000 ppm (C) clams.

3.4.6 Cortisol analyses

The disinfectant treatment (MC-T3 1000 ppm) did not alter the cortisol levels compared to MC-T3 CTRL control and MC-T0 time. No significant changes in the cortisol concentrations were recorded within treatments over time (Fig. 8).

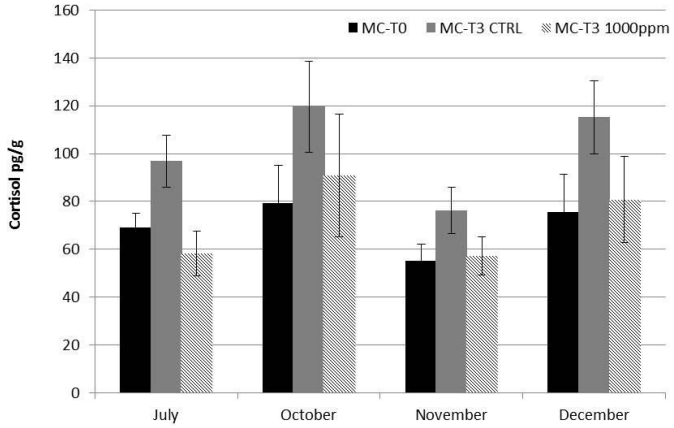

Fig. 8. Digestive gland cortisol levels of Manila clams collected during the depuration assays before (■ MC-T0) and after the disinfectant treatment (■ MC-T3 CTRL ▨ MC-T3 1000 ppm). Data are presented as mean ± SD.

3.4.7 Histopathological analyses

In the trials carried out in July, the histological examinations revealed a fully developed gonadal tissue with a slight predominance of females over males. The 1/3 of untreated clams showed a mild to moderate haemocytic infiltration in gills and interstitium, associated in half of cases the presence of *Perkinsus* sp. In the treated clams (1000 ppm for 3 hours) the situation was similar, except for a case in which there was chronic inflammation with the presence of granulocytomas and a subject in autolysis. In the trials carried out in October, the reproductive system observations showed a clear predominance of males over females by a 2/1 ratio. In this trial, the treatment (1000 ppm for 3 hours) was followed by 1 day of maintenance in the untreated sea water, to verify the possible presence of long-term alterations. However, no noteworthy lesions were observed. Both in treated and untreated in half cases the gills were normal, and the detected branchytis in half cases were associated to *Perkinsus* sp. presence. In 40% of cases, chronic inflammation with the presence of granulocytomas in periglandular connective tissue was detected irrespective of *Perkinsus* presence (Fig. 9). In the trial of November, all genitalia were in involution, anyway 5 females were recognized. Almost all clams had a branchytis with variable severity and in half the cases the

branchytis were associated with the presence of *Perkinsus* sp., with multifocal granulocytomas in the parenchyma. Also in this case there was no difference between the treated (1000 ppm for 3 hours) and untreated subjects. In the December trial, the gonadal tissue was not visible. The clams had mild inflammation in the parenchyma with a mild presence of *Perkinsus* sp., and there was no difference between the treated (1000 ppm for 3 hours plus 1 day maintenance in untreated sea water) and untreated subjects. The presence of lipofuscin was confirmed with Ziehl-Neelsen stain, rare cells showed, in both treated and untreated clams, lipofuscin pigment. Degenerated cells with water or lipid accumulations were rarely observed and no necrosis or apoptosis were present.

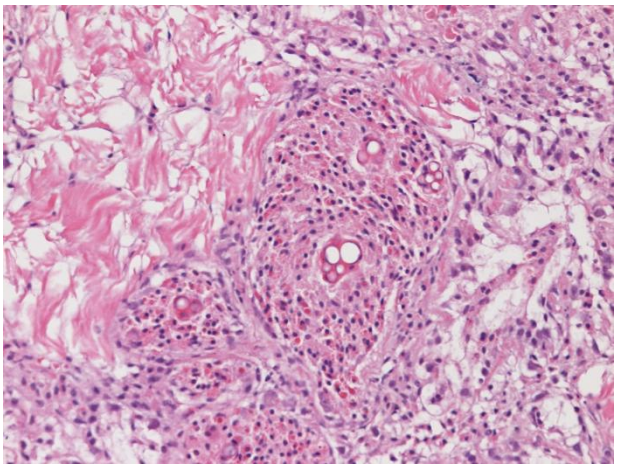

Fig. 9. Clams. Digestive system connective tissue. Cluster of mature trophozoites (encapsulated in well-circumscribed walls forming a cyst-like structure) of *Perkinsus* sp. surrounded by haemocytes forming granulocytomas. EE, 20x.

3.4.8 Sodium dodecylbenzenesulfonate (LAS12) quantification

A first consideration about LAS12 is that it could be commonly present in laboratory solvents and cleaning agents (Rico-Rico et al, 2009); therefore some appropriated precautions were adopted in order to avoid sample contamination (distillation of solvents and heat treatment of glassware at 450°C for 3 hours, successively washed with distilled solvents and the contact with any detergents in the laboratory was strictly avoided.

The analysis of control samples (MC-T0) reported the presence of traces of LAS12, likely due to the contamination of clams before the microbial decontamination process. Clam samples from large retailers were analysed and showed similar contamination levels to MC-T0.

From the analysis of samples, MC-T3 CTRL, MC-T3 1000 ppm and samples from large retailers, showed similar levels of LAS12, all in the range from 200 to 500 ppb.

3.5 DISCUSSION

Depuration and relaying represent essential tools to manage contaminated harvested shellfish, before their access to market (Lees et al., 2010). However, they represent a resource, but also a constraint due to poor effectiveness, limitations and potential risks intrinsic to the processes. The efficacy of depuration strongly depends on the efficiency of the recirculating aquaculture systems (RAS) plant to ensure a good quality of the water (Serratore et al., 2014). Shellfish contribute with their own microflora to microbiologically enrich the water of the system, thus increasing the risk of gathering of pathogens, spreading contamination among different batches and then requiring an effective system to remove or inactivate the microflora released in the water including viruses and V*ibrio* spp., At present depuration treatment is considered satisfactory when these goals are achieved: the reduction of faecal bacteria, in order to comply with the legislative standards, and the control of microbial enrichment in the system (Serratore et al., 2014). The release of microorganisms by shellfish has a negative impact also on the relaying process, as it is carried out in the natural environment. The impact of the release of fish pathogens represents a real risk of transmission of infectious diseases to aquatic animals (Molloy et al., 2013).

For these reasons, a sea water potassium peroxymonosulfate-disinfection process is proposed in the present study to complement and improve the depuration of cultured clams and to improve their microbiological quality in view of their safe placing on the market, movement, depuration, relaying or consumption by humans.

Different reduction values were reported in the literature for bacteria and viruses after MPS-based treatment. Even if the wide variability of treatment conditions applied makes it uneasy to compare the results, it seems clear that successful inactivation performances were obtained against several microorganisms. On these bases MPS-based compounds were ranked as high disinfectant products useful in many different applications (Eleraky et al., 2002; Su & D'Souza, 2012; Martin et al., 2013; Morin et al., 2015).

The MPS-based product tested in the present study shows good bactericidal and virucidal activities in sea water. After having compared several doses and time contacts, the best treatment of contaminated water resulted the use of the 1000 ppm disinfectant concentration for three hours, which reduced about 3 $\log_{10}$ CFU mL^{-1} of *Vibrio* spp. and 3 $\log_{10}$ $TCID_{50}$ mL^{-1} of VNNV in sea water. On the other hand, lower concentrations of disinfectant showed different effects on tested bacterial and viral targets reflecting a different susceptibility of microorganisms to peroxy acids.

Previous studies showed a high resistance of betanodaviruses to a broad range of chemical-physical parameters (Arimoto et al., 1996; Frerichs et al., 2000). Moreover, other viruses, with a similar structure of the viral particles, showed high resistance to peroxy-based compounds, including a MPS-based product (Martin et al., 2013).

Despite the biocidal activity of the proposed treatment (1000 ppm disinfectant treatment - three hours) was equally efficient for the tested bacteria and the virus in sea water, when tested on shellfish a different effect against *Vibrio* spp. and the VNNV was observed.

Accordingly, the significant reduction of the *Vibrio* spp. load in clams produced by the selected treatment (1000 ppm disinfectant for 3 hours), even if less pronounced than that obtained in sea water, as well as the ineffectiveness on VNNV titre in clams, can be explained on the basis of a different susceptibility of these microorganisms to peroxy acids. Additionally, it seems likely that the shellfish itself could protect the micro-organisms against the disinfectant, and/or constitute a physical barrier between the microorganism and the chemicals, thus limiting the amount of disinfectant acting on the micro-organisms and reducing its effectiveness. Accordingly, viruses are not passively accumulated by shellfish and their removal is reduced by a stable link between the virus and the shellfish itself (Maalouf et al., 2010). However, these studies focused their attention on human viruses and up to now no data are available for betanodaviruses. Since recent studies showed that VNNV can be bioaccumulated, restrained, but also released into the water by contaminated clams (Ciulli et al., 2015; Volpe & Ciulli, unpublished data), the proposed treatment can inactivate released virus, thus contributing to reduce the potential risk due to the relaying activity.

As part of the development of the enhanced purification system, it was important to verify the potential effects of the treatment on clam health and welfare as well as the absence of undesired side-effects in the edible flesh. Accordingly, the maintenance of food quality and organoleptic features is an essential requisite for the feasibility of any treatment before commercialization.

The absence of production of peroxidation products and the maintenance of organoleptic features in shellfish subjected to the selected disinfectant treatment, shown by TBARs and VOC pattern analyses, corroborates the suitability of the treatment to improve seafood safety.

By the way, the TBARs analysis showed a clear effect of the sampling, with MDA levels significantly lower in one sampling (July) compared to the others. The higher MDA level at different sampling times may reflect a different dietary input and consequently, a different unsaturation of tissue lipids, in turn affected by phytoplankton features of the environment where the clams were cultivated (Beninger & Stephan, 1985). As a matter of fact, in poikilotherms the molecular strategy leading to homeoviscous adaptation is mainly based on fatty acid unsaturation

which increases as temperature decreases. In turn the unsaturation increase is expected to reflect the dietary input, especially in organisms such as bivalves, which are unable to *de novo* synthesize long-chain polyunsaturated fatty acids (Ventrella et al., 2016). Unsaturated fatty acids are known to be *per se* more prone to oxidative damage than saturated ones (Ayala et al., 2014).

Furthermore, the substantially overlapping VOC pattern in MC-TO, MC-T3 and MC-T3 1000 ppm, irrespective of the exposure to the disinfectant, in the trials of July, October, November and December, clearly points out that, under the conditions tested, the disinfectant treatment could be applied irrespective of the season. To sum up, the results from the two biochemical approaches applied, fully consistent in showing that, under the conditions tested, the selected treatment apparently did not cause any oxidative damage to edible tissues, could reassure consumers about the absence of undesired effects on clam flesh quality.

The presence of cortisol and cortisol-like molecules has been reported in some invertebrates; Ottaviani et al. 1998 found immunoreactive cortisol in molluscan immunocytes, but their direct relationship with stress response is still unclear. The negative correlation between cortisol and population density found by Lagos et al. (2015) and the lack of differences in the hormone levels between controls and treated animals observed in present study make it possible to consider a different pattern of this marker to stress in molluscs, dissimilar to what we usually expected in vertebrates. It is thus necessary to deepen studies to evaluate if cortisol or cortisol-like molecules correlate with the stress level in Manila clam. Anyway, if this is the case, data from the present study are not indicative of stress induction by the disinfection protocol.

Finally, even if some haemocytic infiltrations and chronic inflammation in clam tissues with the presence of granulocytomas, branchytis and *Perkinsus s*p. were detected by histopathological investigations in several clams, such alterations were irrespective of the disinfectant treatment, since they equally occurred in disinfectant-treated and control clams. Therefore, from the shellfish health and welfare point of view, both cortisol and histopathological investigations argue in favour of an unaltered physiological state of the shellfish.

The LAS12 quantification showed a widespread contamination of clams by this product, unrelated to clam origin. The unavoidable presence of LAS in coastal marine ecosystems is a fact, related to detergent discharge via urban sewer systems in the marine medium; in particular, LAS12 settles in costal sediments and accumulates in bivalve shellfish, as clams, that live in close to the seabed and bio-concentrate chemical compounds present in water (Sàez et al., 2000).

Furthermore, analyses of clams, before and after the tested treatment, showed comparable levels of LAS12, always below the concentrations of the few available data (Sàez et al., 2000; Alvarez-

Munoz et al., 2004). These results underline that the contribution of the treatment with the disinfectant to the contamination of the clams should be considered as negligible.

3.6 CONCLUSIONS

The novel Manila clam sea water potassium MPS-based disinfection process set up and tested in the present study clearly improves the microbiological quality of harvested clams with respect to their *Vibrio* spp. load. Furthermore, the proposed treatment greatly reduces the tested bacteria and viruses in the sea water column, being able to counteract possible microorganisms released from shellfish during the depuration process. The proposed treatment reduces the risks related to the shellfish placing on the market, movement, depuration, relaying or consumption by humans. This disinfectant treatment is strengthened by the absence of effects on clam health and welfare and of undesired side-effects or LAS 12 residues in in the edible flesh. Therefore, the proposed treatment may really represent an innovative strategy to face, in a quite easy and feasible way, some still unresolved matter of concern in clam depuration and commercialization.

Acknowledgements

This work was supported by the Department of Veterinary Medical Sciences, University of Bologna, Italy. The Authors would like to thank Dr. Niccolò Guercilena for his assistance in shellfish care and Dr. Elisa Zironi for technical assistance in chemical analyses.

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Final considerations

Bivalve molluscs are well-known bio-accumulators of microorganisms, including finfish and human pathogens. This topic has been long investigated for human pathogens. On the other hand, poor studies were available for finfish pathogens.

The three studies conducted during the Ph.D contribute to expand knowledge on finfish and human pathogens in bivalve molluscs and particularly on betanodavirus presence in bivalve molluscs, on their interaction with the *Redspotted grouper nervous necrosis virus* (RGNNV) and propose a novel method to mitigate bacterial and viral contaminations of bivalve molluscs.

The first study, focusing on the betanodavirus presence in bivalve molluscs, points out that betanodaviruses are widespread in these invertebrates, maybe more than we could expect. Phylogenetic analysis of these viruses shows that strains detected in bivalve molluscs and in finfish are very closely related. Accordingly, betanodaviruses detected in bivalve molluscs in different European countries from 2008 to 2015 mimic the epidemiological patterns of betanodaviruses previously detected in finfish in Southern Europe from October 2000 to November 2009. Moreover, the nucleotide and amino acid sequence analysis of one strain show the existence of a new betanodavirus strain not belonging to any of the already known betanodavirus genotypes. Such a massive and variegate presence of betanodaviruses in bivalve molluscs greatly stresses the risks of transmission to finfish previously feared for other invertebrates. Bivalve molluscs reared in the same area of farmed and wild finfish could accumulate betanodaviruses acting as a reservoir of these viruses. Furthermore, the marketing of alive bivalve molluscs and the relaying activity, allowed by the European regulation, can pose also a real risk of spreading betanodaviruses between different geographical areas.

These data are very relevant considering that these viruses can survive in bivalve molluscs and be released as demonstrated in the second study. Clams, in fact, were demonstrated 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 pose a serious risk for susceptible cohabitant fish species.

The third study proposed a novel method to mitigate the impact of bacterial and viral contaminations in bivalve molluscs. A wider range of microorganisms has been considered for this study, including, not only the VNNV as the most important finfish pathogen, but also the *Vibrio* spp. population naturally associated with sea water that includes several human and finfish pathogens. The novel Manila clam sea water potassium MPS-based disinfection process set up and tested in the present study greatly reduces the tested bacteria and virus loads in the sea water column, being able to counteract the possible microorganisms release from shellfish during the

depuration process. The proposed treatment reduces the risks related to the shellfish placing on the market, movement, depuration, relaying or consumption by humans. This disinfectant treatment is strengthened by the absence of effects on clam health and welfare and of undesired side-effects or LAS 12 residues in the edible flesh. Therefore, the proposed treatment may really represent an innovative strategy to face, in a quite easy and feasible way, some still unresolved matter of concern in clam depuration and commercialization.

The obtained results point out the possible role of bivalve molluscs in the transmission of pathogens to finfish and highlight the needing of surveillance and control activities where a close inter-specific contact is present. The proposed novel disinfection method provides good experimental results and could find wide application in fisheries sector after adequate field tests.

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