



ALMA MATER STUDIORUM  
UNIVERSITÀ DI BOLOGNA

ARCHIVIO ISTITUZIONALE  
DELLA RICERCA

## Alma Mater Studiorum Università di Bologna Archivio istituzionale della ricerca

Spatial and seasonal variability of human and fish viruses in mussels inside and offshore of Ravenna's harbour (Adriatic Sea, Italy)

This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

*Published Version:*

Errani F., Ponti M., Volpe E., Ciulli S. (2021). Spatial and seasonal variability of human and fish viruses in mussels inside and offshore of Ravenna's harbour (Adriatic Sea, Italy). *JOURNAL OF APPLIED MICROBIOLOGY*, 130(3), 994-1008 [10.1111/jam.14806].

*Availability:*

This version is available at: <https://hdl.handle.net/11585/768774> since: 2021-02-27

*Published:*

DOI: <http://doi.org/10.1111/jam.14806>

*Terms of use:*

Some rights reserved. The terms and conditions for the reuse of this version of the manuscript are specified in the publishing policy. For all terms of use and more information see the publisher's website.

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>).  
When citing, please refer to the published version.

(Article begins on next page)

This is the final peer-reviewed accepted manuscript of:

**Errani, F., Ponti, M., Volpe, E., & Ciulli, S. (2021). Spatial and seasonal variability of human and fish viruses in mussels inside and offshore of Ravenna's harbour (Adriatic Sea, Italy). *Journal of applied microbiology*, 130(3), 994–1008.**

The final published version is available online at <https://doi.org/10.1111/jam.14806>

Rights / License:

The terms and conditions for the reuse of this version of the manuscript are specified in the publishing policy. For all terms of use and more information see the publisher's website.

*This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>)*

***When citing, please refer to the published version.***

1 **Spatial and seasonal variability of human and fish viruses in mussels inside and offshore of**  
2 **Ravenna's harbor (Adriatic Sea, Italy)**

3

4 Francesca Errani<sup>a</sup>, Massimo Ponti<sup>b,c</sup>, Enrico Volpe<sup>a</sup>, Sara Ciulli<sup>a\*</sup>

5

6 <sup>a</sup> *Department of Veterinary Medical Sciences, Alma Mater Studiorum, University of Bologna, Viale*  
7 *Vespucchi, 2, 47042 Cesenatico, Italy.*

8 <sup>b</sup> *Dipartimento di Scienze Biologiche, Geologiche e Ambientali (BiGeA) & Centro*  
9 *Interdipartimentale di Ricerca per le Scienze Ambientali (CIRSA), Alma Mater Studiorum,*  
10 *University of Bologna, Via S. Alberto 163, 48123 Ravenna, Italy.*

11 <sup>c</sup> *Consorzio Nazionale Interuniversitario per le Scienze del Mare (CoNISMa), Piazzale Flaminio 9,*  
12 *00196 Roma, Italy.*

13

14 Running head: Virological study in coastal waters

15

16 \* Corresponding author. Tel.: +390547338959.

17 *E-mail address:* [sara.ciulli@unibo.it](mailto:sara.ciulli@unibo.it) (S. Ciulli).

18

19 Sara Ciulli <https://orcid.org/0000-0003-3371-2488>

20 Massimo Ponti <https://orcid.org/0000-0002-6521-1330>

21

22

23

24

25

26

27 **Abstract**

28 **Aims:** This study aims to investigate the presence and spatial-seasonal variability of human and fish  
29 viruses in coastal marine systems using Ravenna's harbor area (Adriatic Sea, Italy) as a model.

30 **Methods and Results:** Human viruses (noroviruses and hepatitis A virus) and one of the most  
31 threatening finfish pathogens, the nervous necrosis virus (NNV), were investigated in mussels  
32 living inside and offshore Ravenna's harbor. Thirty-three and 36.7% of tested mussel samples  
33 resulted contaminated by human and fish viruses respectively. A different spatial-seasonal  
34 distribution was observed. Human viruses were detected mainly in inner port sites during colder  
35 months, while NNV was detected in both inside and offshore of Ravenna's harbor, mainly during  
36 warmer months.

37 **Conclusions:** The presence of human viruses in the inner port close to the city center could be  
38 attributed to wastewaters carrying pathogens in the port environment and this arises public health  
39 concerns, however the presence of these viruses limited to the canal port during the winter can  
40 greatly reduce the risk to human health. Regarding NNV, the accumulation and release of viable  
41 virus by mussels, could represent a viral source for susceptible finfish. These findings reflect the  
42 different epidemiological features of these infections and indicate the importance to choose the  
43 correct indicator to monitor viral contaminations.

44 **Significance and Impact of the Study:** The high frequency of viral contamination pointed out in  
45 the study stresses the imperative to monitor the viral presence in all coastal habitats where the high  
46 natural value meets several recreational and commercial activities such as the Ravenna's harbor  
47 area. Particularly, this study could represent a novel starting point for the development of a more  
48 structured bio-monitoring program, in order to ensure improved environmental management and  
49 safety of coastal areas.

50

51 **Keywords:** Viral contamination; Mussel; Harbor; Norovirus; Hepatitis A virus; Nervous Necrosis  
52 Virus; Adriatic Sea.

53

## 54 **Introduction**

55 Viruses are the most abundant members of the marine ecosystems (Munn, 2006) and it has been  
56 estimated that the world's oceans may contain in the order of  $10^8$  viruses  $\text{mL}^{-1}$  (Middleboe and  
57 Brussad, 2017). They play an enormous role in ocean processes through their interactions with all  
58 types of marine organisms. Accordingly, they can infect organisms ranging from the smallest kind,  
59 like marine bacteria and Archaea, to the largest marine mammals (Munn, 2006). Some of them are  
60 recognized as the causative agents of fish diseases such as nervous necrosis viruses (NNV) being  
61 responsible for the viral encephalo-retinopathy (VER) in several marine finfish species (Doan et al.  
62 2017; Middleboe and Brussaard, 2017). The VER, also known as viral nervous necrosis, is  
63 characterized by a vacuolating necrosis of nervous tissue and a mortality rate up to 100%, causing  
64 severe losses in finfish farming, but also damaging the natural finfish stocks (Vendramin et al.  
65 2013; Doan et al. 2017; Volpe et al., 2020). The NNVs are small single stranded positive-sense  
66 RNA virus of the genus *Betanodavirus*, family *Nodaviridae* and their genome consists of two  
67 molecules of RNA; the RNA1 which encodes a non-structural protein with RNA dependent RNA  
68 polymerase (RdRp) and RNA2 which encodes the coat protein (CP; Doan et al. 2017). Based on a  
69 partial nucleotide sequence of the coat protein gene, NNVs are divided into 4 genotypes: Striped  
70 jack nervous necrosis virus (SJNNV), Tiger puffer nervous necrosis virus (TPNNV), Barfin  
71 flounder nervous necrosis virus (BFNNV) and Redspotted grouper nervous necrosis virus  
72 (RGNNV; Doan et al. 2017). Furthermore, reassortant strains have emerged from the reassortment  
73 between RGNNV and SJNNV genotypes, then reported as RGNNV/SJNNV (containing the RNA1  
74 deriving from the RGNNV genotype and the RNA2 originating from the SJNNV genotype) and  
75 SJNNV/RGNNV (containing the RNA1 deriving from the SJNNV genotype and the RNA2  
76 originating from the RGNNV genotype; Toffolo et al. 2007; Panzarin et al. 2012).

77 The sea can also act as a source for the transmission of viruses of human origin, especially the  
78 enteric viruses, which contaminate coastal waters (Munn, 2006). They can cause a broad spectrum

79 of human medical conditions, including hepatitis, gastroenteritis, meningitis, fever, rash and  
80 conjunctivitis (Maalouf et al. 2010). Viruses more frequently implicated in these outbreaks are  
81 noroviruses and the hepatitis A virus (Maalouf et al. 2010; Serratore et al., 2014). Noroviruses  
82 (NoV) are very stable in the environment and they are the most common cause of human acute viral  
83 gastroenteritis causing both sporadic and endemic illnesses across all age groups (Serratore et al.,  
84 2014; Ilic et al. 2017). NoV are non-enveloped single stranded positive-sense RNA viruses of the  
85 genus *Norovirus*, family *Caliciviridae* (Ilic et al. 2017). They are highly diverse and are divided into  
86 seven genogroups (GI-GVII) of which the most frequently found among people are GI and GII (van  
87 Beek et al. 2018). Furthermore, each genogroup can be divided in several genotypes based on the  
88 analysis of the RNA-dependent RNA polymerase (RdRp). Particularly, 14 GI and 29 GII genotypes  
89 have been described so far (Medici et al. 2014).

90 Hepatitis A virus (HAV) is a hepatotropic agent from the genus *Hepatovirus*, family *Picornaviridae*  
91 and is responsible for acute viral hepatitis worldwide, whose transmission is linked to sanitary and  
92 living conditions of the population (Mbayed et al. 2002; Chironna et al. 2003; Maalouf et al. 2010).  
93 The HAV has been classified in seven different genotypes (I-VII) and several subtypes (Mbayed et  
94 al. 2002). The subgenotype IA is the most widespread in the world (Mbayed et al. 2002; Chironna  
95 et al. 2003).

96 Mussels have been proposed to be useful for biomonitoring trace levels of contaminants in coastal  
97 waters. In fact, they are characterized by a wide distribution, sessile lifestyle, easiness of sampling,  
98 resistance to stress and high accumulation of a wide range of microorganisms including human and  
99 fish pathogens (Goldberg et al. 1978; Croci et al. 2007; Serratore et al. 2014; Ilic et al. 2017; Volpe  
100 et al. 2018).

101 The aim of this study was to investigate the presence of environmental contamination by human and  
102 fish viruses inside and offshore of Ravenna's harbor monitoring mussels as bio-vectors.

103 Particularly, the presence of human viruses like NoV and HAV and the presence of one of the most  
104 threatening finfish pathogens, the NNV, was investigated considering their spatial and seasonal

105 variability. Based on the results of this study, possible implications for human health and  
106 aquaculture have been discussed.

107

## 108 **Materials and method**

### 109 **Study area**

110 Ravenna is the major coastal city of Emilia-Romagna region (northwest Adriatic Sea) and one of  
111 the largest commercial seaports in Italy. The harbor is structured as a major canal port extending for  
112 11 km from the center of Ravenna to the touristic seacoast (Airoldi et al. 2016). The seaside is  
113 protected by two converging dams, each 2,400 meters long, while the side towards the city is close  
114 to the railway station (Airoldi et al. 2016). The canal port is also directly connected with two  
115 surrounding brackish lagoons (Pialassa Baiona and Pialassa Piomboni) which are comprised of the  
116 southern part of the Po Delta Interregional Park and included in the list of wetlands of international  
117 importance under the Ramsar Convention (Ponti et al. 2011). The Emilia-Romagna's coastal areas  
118 are naturally sedimentary. Shallow subtidal sediments are comprised of well sorted fine to medium  
119 sand and are colonized by macrofaunal assemblages generally dominated by bivalve mollusks  
120 (Airoldi et al. 2016).

121 In front of the harbor, offshore gas platforms introduce artificial hard bottoms which are colonized  
122 by sessile invertebrates and vagile fauna that vary according to depth and exposure to prevailing  
123 currents (Ponti et al. 2002). Mussels dominate the benthic assemblages from the surface down to 12  
124 m, the usual maximum depth of the summer thermocline, while below oysters, cnidarians and  
125 sponges are the most abundant taxa (Ponti et al. 2002). These structures also act as fish aggregating  
126 devices, leading to enrichment and greater diversification of the local fish assemblages (Fabi et al.  
127 2004).

128 The port of Ravenna is one of the most important ports in Italy with intensive naval traffic,  
129 including commercial, touristic and recreational activities (Airoldi et al. 2016; Ravenna Port

130 Authority<sup>1</sup>). Nowadays, following coastal and tourism development and environmental degradation,  
131 the role of fishery in the regional economy is reduced, however artisanal fisheries still represent an  
132 important revenue for the local communities (Pranovi et al. 2016). The main fishing activity in the  
133 open sea includes the harvesting of marine clams (*Chamelea gallina*) by using hydraulic dredges  
134 and cuttlefish (*Sepia officinalis*) by fish traps, offshore mussel farms and the harvesting of mussels  
135 (*Mytilus galloprovincialis*) from offshore platforms by surface supplied-divers and fishery by  
136 trawling (Airoldi et al. 2016; Pranovi 2016). The small local fishery in the lagoons includes the  
137 harvesting of the non-indigenous Manila clams (*Ruditapes philippinarum*; Abbiati et al. 2010; Ponti  
138 et al. 2017). Moreover, some fish farms are present in other lagoons along the Adriatic coast, of  
139 which the closest is the Valli di Comacchio lagoon complex; however, no direct water connection  
140 exists between these lagoons and the Ravenna's harbor. In these lagoons, the main reared finfish  
141 species are European seabass (*Dicentrarchus labrax*), gilthead seabream (*Sparus aurata*) and  
142 European eel (*Anguilla anguilla*; Ponti et al. 2007; Abbiati et al. 2010).

143

#### 144 **Sampling design and field activities**

145 Mussels were identified as the most abundant and accessible bivalve mollusks suitable for virus  
146 detection in the whole study area. Accordingly, mussel samples (*M. galloprovincialis* and  
147 *Xenostrobus securis*) were collected to investigate the presence of human (hepatitis A virus,  
148 norovirus) and fish (nervous necrosis virus) viruses.

149 The sampling design aimed to investigate three areas with different environmental features: the  
150 inner port (St. 5 and St. 6), at 11.3-12.6 km from the port entrance, the outer port (St. 1 and St. 2),  
151 1.2-2.8 km from the port entrance, and the offshore area (gas platforms; Fig. 1).

152 A year-round sampling (March 2018-March 2019) was conducted with mussel collection every two  
153 months in inner and outer port sites. During the summer period (July and September) mussel

---

<sup>1</sup> <http://www.port.ravenna.it/anno-2017>

154 samples were also collected from offshore gas platforms, randomly chosen at 16-30 km from the  
155 port entrance (labeled gas platforms; Fig. 1). In summer, in fact, gas platforms are subjected to  
156 periodical cleaning by removing mussels from the support pylons, these mussels are sold for the  
157 human consumption.

158 After collection, mussel samples were maintained under refrigerated conditions and immediately  
159 transferred to the laboratory to be processed for viral detection.

160 During each sampling, physical-chemical water parameters (temperature, salinity, pH, oxidation /  
161 reduction potential, dissolved oxygen) have been collected. Temperature and salinity were  
162 measured at the time of the sampling using a conductivity meter-thermometer (HD9213, Delta  
163 OHM). Where practicable (St. 1 and St. 6) mean daily temperature values were also collected using  
164 data loggers set to store a data every 10 minutes (Star-Oddi, DST Centi-T). This measurement was  
165 performed to obtain a more accurate and less biased, by the time of the sampling, temperature  
166 values. The mean daily temperature values were also collected to compare the difference between  
167 the two further apart sites within the canal port (St. 1 and St. 6). The pH and oxidation / reduction  
168 potential (ORP) were measured using a pH meter (HI98121, Hanna Instrument). Some pH, ORP  
169 and oxygen data have been not available due to instrument failures.

170

### 171 **Virus detection and genotyping**

172 Each sample consists of at least 10 mussel specimens belonging to one species: *M. galloprovincialis*  
173 was collected from offshore gas platforms, St. 1, and St. 2 whereas the non-indigenous *X. securis*  
174 was collected from St. 5 and St. 6. The latter species, original from Australia and New Zealand  
175 (Occhipinti-Ambrogi, 2000), in the inner port replaced the native species, almost similar in size and  
176 shape. Despite no comparative data are available on viral accumulation of these two bivalve  
177 species, they are alternatively used as bioaccumulation indicators in monitoring program where is  
178 not viable to use a single bivalve (e.g. Markich and Jeffree, 2019). Hepatopancreas tissues were  
179 pooled, homogenized and treated with proteinase K (Sigma, St. Louis, MO, USA). RNA was

180 extracted from a 100 µl supernatant aliquot, using the commercial kit NucleoSpin RNA (Macherey-  
181 Nagel, Düren, Germany) according to the manufacturer's instruction. RNA samples were stored at -  
182 80 °C until use.

183 The presence of hepatitis A virus (HAV) was investigated via an RT-nested PCR assay targeting a  
184 VP1 fragment performed according to a method previously described (Le Guyader et al. 1994). To  
185 detect the presence of norovirus genogroup I (NoV GI) and genogroup II (NoV GII) two real time  
186 RT-PCR assays previously described were performed (Suffredini et al. 2008). The method  
187 performed enables qualitative detection of NoV GI and GII RNA in the tested samples, which were  
188 considered positive when a Ct value below 44 was present. In case NoV GI or GII was detected,  
189 two RT-nested PCR assays were conducted to amplify an RdRp gene fragment using protocols  
190 previously described (Vinjé and Koopmans 1996; Green et al. 1998; Boxman et al. 2006). The  
191 presence of nervous necrosis virus (NNV) was investigated via two RT-PCR assays followed by  
192 nested PCRs targeting viral RNA1 and RNA2 performed according to methods previously  
193 described (Toffolo et al. 2007; Volpe et al. 2018). Samples positive to at least one of the two PCR  
194 reactions (RNA1 or RNA2) were considered positive to NNV. Details of primers and probes used  
195 are reported in table 1. Positive and negative controls were run along with all reactions.

196 PCR products of samples positive to any of the RT-PCR or nested PCR were purified using the  
197 Exosap reagent (Affymetrix, Santa Clara, USA) and then sequenced by the Bio-Fab Research srl  
198 (Rome, Italy). The sequences obtained were corrected manually and analyzed through the online  
199 software Basic Local Alignment Search Tool (BLAST), available on the National Center for  
200 Biotechnology Information site<sup>2</sup>, to confirm the viral identity.

201 To further genotype HAV strains detected in the study, a phylogenetic analysis was conducted.  
202 Partial VP1 gene sequences were aligned and compared with HAV sequences of the reference strain  
203 HM-145 and with a selection of Italian HAV strains (Chironna et al. 2003) available in GenBank<sup>3</sup>

---

<sup>2</sup> <http://blast.ncbi.nlm.nih.gov/Blast.cgi>

<sup>3</sup> [www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)

204 using Clustal W implemented in the BioEdit software<sup>4</sup>. Neighbor-joining phylogenetic analysis of  
205 the partial VP1 gene was performed with MEGA 7 software<sup>5</sup>. Bootstrap analysis was carried out on  
206 1,000 replicates.

207 The genotyping of norovirus detected in mussels was conducted using the Norovirus Typing Tool  
208 Version 2.0 (Kroneman et al. 2011) analyzing an RdRp gene fragment.

209 For NNV detected in this study, a phylogenetic analysis was conducted. RNA1 and RNA2  
210 nucleotide sequences were aligned and compared with NNV sequences detected in both marine  
211 vertebrates and invertebrates as well as with betanodavirus reference strains available in GenBank<sup>3</sup>  
212 using Clustal W implemented in the BioEdit software<sup>4</sup>. Maximum-likelihood phylogenetic analysis  
213 of partial RNA1 and RNA2 sequences was performed with MEGA 7 software<sup>5</sup>. Bootstrap analysis  
214 was carried out on 1,000 replicates.

215

#### 216 **Data availability**

217 The viral sequences obtained in this study were deposited into the GenBank database and are  
218 available under the following accession numbers MT755963, MT758315-MT758420, MT759744-  
219 MT759760.

220

#### 221 **Statistical analyses**

222 A paired-sample t-test (two-tailed; Prism version 6.0 software, GraphPad Software, San Diego,  
223 USA) was used to compare mean daily temperature values collected at St. 1 and St. 6. The level of  
224 statistical significance acceptance was  $p < 0.05$ .

225 Chi-square or Fisher's exact tests were used to correlate the viral presence/absence to seasonality  
226 and sampling sites (Prism version 6.0 software, GraphPad Software, San Diego, USA). Analysis  
227 was conducted independently for human viruses and the finfish virus (NNV). Regarding

---

<sup>4</sup> <http://bioedit.software.informer.com/>

<sup>5</sup> [www.megasoftware.net](http://www.megasoftware.net)

228 seasonality, three groups were made based on month average temperature recorded at sampling  
229 times: cold season (month average temperature  $<15^{\circ}\text{C}$  January and March), mid-season (month  
230 average temperature  $15\text{-}20^{\circ}\text{C}$ , May and November) and warm season (month average temperature  
231  $>20^{\circ}\text{C}$ , July and September). Regarding sampling sites, three groups were made based on site  
232 location: offshore gas platforms, outer port sites (St. 1 and St. 2) and inner port sites (St. 5 and St.  
233 6). The level of statistical significance acceptance was  $p < 0.05$ .

234

## 235 **Results**

### 236 **Physical-chemical parameters**

237 Within the Ravenna's canal port, the water temperature measured at mussel sampling times showed  
238 values ranging between  $7.0^{\circ}\text{C}$  and  $28.1^{\circ}\text{C}$  (Fig. 2a). Regardless the site position, the temperature  
239 values of all the sampling sites presented a high variability depending on the seasons with the  
240 highest temperature in July and the lowest temperature in January. The salinity showed values  
241 ranging between 18.6 psu and 27.4 psu (Fig. 2b). Salinity fluctuations were mainly regulated by the  
242 seawater tidal exchange and freshwater runoff. The pH presented values ranging between 7.80 and  
243 8.78 (Fig. 2c). The ORP showed always water oxidizing conditions with values ranging between 18  
244 mV and 145 mV (Fig. 2d). The concentration of dissolved oxygen ranged between 4.9 mg/L and 8.9  
245 mg/L and between 65% and 109% of saturation. All physical-chemical water parameters collected  
246 within the Ravenna's canal port at mussel sampling times are reported in the supplementary  
247 materials (Table S1).

248 Mean daily water temperatures collected at outer port (St. 1) and inner port (St. 6) sites showed  
249 values ranging between  $7.0^{\circ}\text{C}$  and  $31.0^{\circ}\text{C}$  (Fig. 3). The mean daily temperature difference between  
250 the two sites ( $\Delta T_{\text{St. 6} - \text{St. 1}}$ ) ranged between  $-1.79$  and  $2.92^{\circ}\text{C}$  and, on average, the inner site (St. 6)  
251 was  $0.99^{\circ}\text{C} \pm \text{SE } 0.07$  ( $p < 0.001$ ,  $n=176$ ) warmer than the external one (St. 1).

252

### 253 **Human viruses' detection and genotyping**

254 Detailed results concerning human virus contaminations detected in mussels are reported in table 2.  
255 Thirty-three percent (10/30) of tested samples resulted contaminated with at least one of the  
256 investigated human viruses. In particular, two samples were contaminated with HAV (6.7%) and 10  
257 (33%) were contaminated with NoV GI and/or GII. Six samples were contaminated with more than  
258 one virus. Particularly, two samples were contaminated with both HAV and NoV and six samples  
259 presented both NoV GI and NoV GII contaminations.

260 Regarding spatial variability, no viral contamination was detected in mussel samples collected from  
261 the gas platforms, whereas 38.5% (10/26) of mussel samples collected from Ravenna's port (St. 1,  
262 St. 2, St. 5 and St. 6) resulted contaminated with HAV and/or NoV. Particularly, 61.5% (8/13) of  
263 the samples collected from the inner sites (St. 5 and St. 6) showed to be contaminated (HAV n=2;  
264 NoV n=8), whereas only 15.4% (2/13) of those collected from the outer sites (St. 1 and St. 2)  
265 presented viral contamination (NoV). Contamination of mussels in inner port resulted significantly  
266 higher compared to outer port ( $p=0.04$ ). Concerning the platform area, the unviability of the gas  
267 platform sampling during cold months, due to current policy, could have affected the prevalence of  
268 viruses in samples collected in this area.

269 Regarding seasonal variability, a significant difference in contamination prevalence was observed  
270 among seasons ( $p=0.002$ ); particularly none of the mussel samples collected in July and September  
271 (warm season) resulted contaminated. On the contrary, 70% (7/10) of tested samples in the cold  
272 season (March and January) and 37.5% (3/8) of those collected in the mid-season (May and  
273 November) were contaminated.

274 A VP1 gene fragment was sequenced for HAV detected in mussels sampled in March 2018 and  
275 May 2018. The two sequences showed a 100% nucleotide identity. The detected HAV strain  
276 exhibited the highest nucleotide identity (98-99%) with sequences of HAV subtype IA (Genbank  
277 accession numbers: MF416223; AY441441; AJ505803; AJ505800). Phylogenetic analysis of HAV  
278 strains detected in this study confirmed that they belong to the subtype IA (Fig. 4).

279 The use of two genogroup-specific real time RT-PCR assays showed the presence of both NoV GI  
280 and NoV GII genogroups in tested samples. Considering all samples positive to NoV (n=10), 60.0%  
281 (n=6) of contaminated mussels presented NoV GI and 100% presented NoV GII. Most of the  
282 samples (n=6), in fact, showed to be contaminated with both GI and GII genogroups.  
283 For six samples, an RdRp fragment was sequenced and characterized to genotype level. Concerning  
284 genogroup I, the presence of the genotypes GI.P2 (n=2) was detected, whereas regarding genogroup  
285 II, genotypes GII.P4 (n=1), GII.P17 (n=1), GII.P21 (n=1) and GII.Pe (n=1) were detected. The  
286 genotyping showed a high variability of NoV variants in the investigated area.

287

### 288 **Nervous necrosis virus detection and genotyping**

289 Detailed results concerning NNV contamination detected in mussels are reported in table 3. The  
290 NNV was detected in 36.7% (11/30) of tested samples. Regarding spatial variability 30.8% (4/13)  
291 of samples collected from the inner sites (St. 5 and St. 6), 38.5% (5/13) of those collected from the  
292 outer sites (St. 1 and St. 2) and 50% (2/4) of samples collected from gas platforms showed to be  
293 contaminated with NNV. No significant difference was pointed out in contamination prevalence  
294 detected in different sampling sites. Considering the number of positivity per season, 20% (2/10) of  
295 the mussel samples collected in cold season (January and March) , 25% (2/8) of samples collected  
296 in mid-season (May and November) and 58.3% (7/12) of those collected in warm season (July and  
297 September) were contaminated, however these differences were not significant.

298 Sequences of a fragment of RNA1 and/or RNA2 were obtained from all positive samples.

299 The phylogenetic analysis of the RNA1 showed that NNV detected in mussels clustered in various  
300 subgroups of RGNNV genotype. Furthermore, it was possible to detect a contamination from two  
301 different NNV viral strains in two samples collected in July and September (Fig. 5).

302 The phylogenetic analysis of RNA2 showed that most of the NNVs from bivalve mollusks clustered  
303 within RGNNV genotype, however, two viruses detected in mussels collected in March and May

304 2018 clustered within the SJNNV genotype (Fig. 6), suggesting the presence of a RGNNV/SJNNV  
305 reassortant or corresponding parental strains.

306

## 307 **Discussion**

308 The Ravenna's harbor is an area of cultural and economic value (Airoldi et al. 2016). Historically  
309 high anthropogenic pressure on the coast has led to severe urbanization and overexploitation of  
310 natural resources (Airoldi and Beck 2007). Accordingly, this area suffers several problems, which  
311 are typical of the urbanized environments, including loss of habitats, loss of species, introduction of  
312 non-indigenous species, pollution and poor water quality (Airoldi et al. 2016). The area of the  
313 Ravenna's harbor and the connected coastal lagoons receive several civil and industrial wastewaters  
314 carrying nutrients, different types of pollutants and cooling waters from several industrial plants.  
315 Although nowadays discharges comply with the current laws, their accumulation still raises  
316 concerns (Ponti et al. 2009; 2011). Enteric viruses such as hepatitis A virus and noroviruses  
317 originating from human excreta may enter into the environment through the discharge of waste  
318 materials from infected individuals (Maalouf et al. 2010).

319 The virological investigation conducted in this study showed the presence of several viruses in the  
320 inside and offshore waters of Ravenna's harbor and this also confirms the usefulness of mussels as  
321 an effective tool for monitoring human and fish viruses in seawater.

322 Particularly, 33% of the analyzed samples were contaminated with human pathogens (HAV and  
323 NoV). Similarly, previous surveys reported a high prevalence (22-51.4%) of NoV in shellfish  
324 collected in the Adriatic Sea (Crocì et al. 2007; Suffredini et al. 2012). However, in our study, the  
325 presence of both human viruses (HAV and NoV) was found only inside the Ravenna's canal port,  
326 while no human viruses have been found in mussels collected from the offshore gas platforms.  
327 Despite this result can be influenced by the limited number of samples, a dilution effect due to  
328 distance from the coast might be the main cause of this outcome (Maalouf et al., 2010).

329 Furthermore, a significant diverse degree of contamination was observed in the inner and the outer

330 port with mussels from the inner sites (St. 5 and St. 6) presenting a higher contamination rate  
331 (61.5%) compared to the outer sites (St. 1 and St. 2) (15.4%). These findings suggest that the  
332 presence of human viruses in the area closest to the city center could be attributed to the presence of  
333 sewage drains and untreated wastewaters that may introduce these pathogens in the most inner port  
334 environment, as occurred in other geographic areas (Maalouf et al. 2010; Henigman et al. 2015;  
335 Gonçalves et al. 2018). Previous studies conducted in this area showed a significant impact on  
336 macrobenthic invertebrate populations due to the inputs of wastewater from urban and industrial  
337 sewage treatment plants and cooling water from power plants (Ponti et al. 2009; 2011) showing a  
338 correlation between environmental and viral contaminations in bivalves.

339 Different bivalve species were used for the surveillance with a higher amount of *M.*  
340 *galloprovincialis* in platform and outer port samples and a higher amount of *X. secures* in inner port  
341 samples. Unfortunately, no comparative data are available on viral accumulation of these two  
342 bivalve species; however, they are alternatively used as bioaccumulation indicators in monitoring  
343 program where is not viable to use a single bivalve suggesting the comparability of data obtained  
344 from different species (e.g. Markich and Jeffree, 2019).

345 Regarding HAV, the strain detected in this study belongs to the subtype IA. The genotype HAV I is  
346 considered the most prevalent worldwide and particularly, the subtype IA is more widespread than  
347 the subtype IB; genotyping and molecular epidemiology of HAV have been used to identify  
348 geographic or epidemiological sources of HAV isolates (Mbayed et al. 2002). The HAV IA strains  
349 have been frequently isolated from human infections associated with the consumption of raw  
350 seafood in southern Italy (Chironna et al. 2003).

351 Also for NoV, the genogroup and genotype determination have been useful to monitor the global  
352 spread of noroviruses (Henigman et al. 2015; van Beek et al. 2018). Furthermore, genotype profiles  
353 may help to distinguish the origin of the outbreaks; the NoV genogroup I (GI) represents the most  
354 frequently implicated in shellfish and water-related outbreaks (Maalouf et al. 2010). Conversely,  
355 NoV genogroup II (GII), and particularly genotype GII.4 has been recognized as the one most often

356 associated with person-borne outbreaks (Verhoef et al. 2015). In this study, both genogroups (GI  
357 and GII) have been found. Genetic characterization showed the presence of genotypes NoV GI.P2,  
358 which has already been described in mussels collected from harvesting areas in Slovenia and  
359 associated with the consumption of raw seafood in China (Henigman et al. 2015; Wang et al. 2015).  
360 Regarding NoV GII, genotyping has shown the presence of GII.P4, GII.P17, GII.P21 and GII.Pe. A  
361 previous study pointed out the presence of several norovirus GII.P4 and GII.P21 in mussels  
362 collected in Slovenia (Henigman et al. 2015). At present, GII.Pe, GII.P4 and GII.P17 represent the  
363 most frequent norovirus genotypes detected worldwide (van Beek et al. 2018) and in this respect,  
364 our virological investigation using mussels of Ravenna's harbor reflects this scenario.

365 The virological investigation also showed HAV/NoV and NoV GI/NoV GII mixed contaminations  
366 in the same sample. Mixed viral contaminations in shellfish have already been described in several  
367 studies and associated with the presence of human pollution in mussel farming areas (Crocini et al.  
368 2007; Ilic et al. 2017). Moreover, for the first time a mixed contamination with both human and fish  
369 viruses was detected in bivalve mollusks.

370 Regarding seasonal variability, it is worth noting that, bivalve mollusks during cold months  
371 accumulate a greater amount of microorganisms and with them, viruses (Lipp et al. 2001). This fact,  
372 coupled with the enhanced survival of viruses at lower temperatures may explain the seasonal  
373 increase of norovirus outbreaks and high contamination levels of wastewaters by enteroviruses  
374 during the cold period (Burkhardt et al. 1992; Maalouf et al. 2010; van Beek et al. 2018).

375 In this study norovirus contaminations have been detected in mussels collected in March 2018,  
376 May, November, January and March 2019 whereas no viral contaminations have been detected in  
377 July and September showing a significant more frequent contamination in cold months similarly to  
378 what was observed in other Adriatic areas (Ilic et al. 2017; La Bella et al. 2017; Gonçalves et al.  
379 2018).

380 Overall, the findings of NoV and HAV in mussels in the investigated area could represent a risk to  
381 human health. Although fishing and bathing are prohibited in the port, it must be taken into

382 consideration that the canal port is strictly connected with the surrounding lagoons, where fishing is  
383 carried out, and with the littoral where, mostly during the summer innumerable nautical and  
384 recreational activities take place, including illegal harvest of mussels for personal consumption  
385 from the breakwaters. Previous studies have shown that the pathogens discharged from wastewaters  
386 pose a health risk to everyone exposed to the polluted waters, principally among recreational users  
387 (Wyn-Jones et al., 2011; Gonçalves et al. 2018). However, spatial and seasonal analysis showed  
388 that human viral contaminations in the Ravenna's harbor are limited to the canal port during the  
389 winter, which can greatly limit the risk to human health. Indeed, recreational fishing, which are  
390 allowed only from the outer port dams, is less frequent in winter.

391 Mussels collected from Ravenna's harbor were also frequently contaminated by the finfish  
392 pathogen NNV. The NNV has been previously detected in several marine invertebrates including  
393 bivalve mollusks in the Mediterranean Sea and Eastern Asia (Ciulli et al. 2010; Panzarin et al. 2012;  
394 Kim et al. 2018; Volpe et al. 2018); however, these species cannot be considered susceptible hosts,  
395 as NNV replication has not been demonstrated in these animals. Nevertheless, experimental  
396 contamination trials, conducted with clams, pointed out bivalve mollusks are able not only to  
397 accumulate NNV (Ciulli et al. 2017), but also to release viable viral particles posing concerns about  
398 their possible role as virus carriers (Volpe et al. 2017).

399 A previous study showed a high prevalence (26.3%) of NNV contamination in retail bivalve  
400 mollusks collected in different European countries (Volpe et al. 2018). Similarly, the virological  
401 investigation conducted in the inside and offshore waters of Ravenna's harbor showed a high  
402 percentage of contamination in tested mussels (30-50%) independently from the site of collection  
403 (inner and outer port sites and offshore gas platforms). These mussels could have accumulated  
404 NNV released by farmed finfish during VER outbreaks, however, despite some finfish farms with  
405 NNV susceptible species are located in lagoons northern to Ravenna (Ponti et al. 2007), these  
406 brackish water are not directly connected to the Ravenna's port and its surrounding lagoons. Several  
407 hypotheses can explain the detection of NNV, in an area free of finfish farms rearing susceptible

408 species. NNV could be moved by cargo ships via ballast water and carried by biofouling,  
409 considering that Ravenna is one of the most important ports in Italy, where commercial and touristic  
410 naval traffic are particularly intense. Previous studies have focused on issues related to naval  
411 transport showing that the discharge of water, sediment and biofilm from ballast water tanks of  
412 ships is a prominent vector of aquatic invasive species, pathogens including viruses and toxic  
413 species to coastal regions (Drake et al. 2007; Kim et al. 2016).

414 Moreover, breakwaters, jetties and other artificial structures, which are so abundant along the  
415 northwestern Adriatic coast act as ecological corridors (Airoldi et al. 2015). Accordingly, these  
416 artificial structures, which are colonized by mussels, may have a potential role also in marine  
417 viruses spread.

418 Genotyping of viruses detected in this study could contribute to the understanding of their origin.  
419 The detected NNVs resulted to be mainly RGNNV genotype. This is the most widespread NNV  
420 genotype across the Mediterranean Basin. Furthermore, at the phylogenetic analysis the detected  
421 viruses clustered with NNV strains previously detected in finfish and bivalve mollusks of the  
422 Adriatic Sea (Panzarin et al. 2012; Volpe et al. 2018). The presence of a putative reassortant strain  
423 RGNNV/SJNNV was also detected in mussels of the Ravenna's harbor. Reassortant NNV strains  
424 have emerged from the reassortment of genotypes RGNNV and SJNNV and, so far, they have been  
425 detected mainly in the Mediterranean Basin (Toffolo et al. 2007; Oliveira et al. 2009; Panzarin et al.  
426 2012; Volpe et al., 2020). These findings pointed out that NNVs contaminating mussels from  
427 Ravenna's harbor seem to be autochthonous strains and they suggest that these viruses could  
428 originate from sources different from ballast water. The NNV, in fact, could be directly released in  
429 this area by infected native marine finfish species. NNV, in fact, is able to replicate in cells of  
430 permissive hosts and to be released at high titers in the water. Viral replication is strongly  
431 influenced by several factors such as temperature and fish density. Accordingly, disease outbreaks  
432 caused by NNV are mainly described in farmed finfish during summer (Doan et al. 2017). As no  
433 farms rearing susceptible finfish species are present in the investigated area, nor in the lagoons

434 directly connected to the port, we hypothesized that the NNV could be released directly by infected  
435 wild marine finfish species. NNV, in fact, has been previously isolated from several asymptomatic  
436 wild marine finfish species (Ciulli et al. 2007). Furthermore, experimental trials have demonstrated  
437 that asymptomatic finfish can transmit the infection to susceptible host (Doan et al. 2017). The  
438 frequent presence of NNV shown in this study in mussels from inside and offshore waters of  
439 Ravenna's harbor suggests that susceptible native finfish host species could be infected and release  
440 the virus in the water at high titers during summer. This hypothesis seems to be reinforced by the  
441 observation that most of the NNV contaminated mussels have been detected during the July and  
442 September sampling. A previous survey on NNV shellfish contamination showed a higher detection  
443 rate of RGNNV in summer than in winter, which was similar to the known seasonal patterns of  
444 finfish infection (Kim et al. 2018). Furthermore, experimental trials demonstrated that some bivalve  
445 mollusk species are able to accumulate and release viable viruses, including NNV (Molloy et al.  
446 2013; Volpe et al. 2017; Kim et al. 2018). The accumulation and release of viable NNV by mussels  
447 in Ravenna's harbor area could represent a viral source for other wild susceptible finfish hosts,  
448 enabling to complete the epidemiological cycle of NNV infection in the natural environment.  
449 To sum up, virological investigation in inside and offshore waters of Ravenna's harbor showed the  
450 presence of several viral contaminations in mussels. Despite the study applied a qualitative  
451 methodology without quantifying viral loads, it permitted to successfully detect human and fish  
452 virus contaminations in mussels and to evaluate their frequency and distribution. A different spatial  
453 and seasonal distribution were observed in human and fish virus contaminations. Human viruses  
454 were detected mainly in inner port sites during colder months suggesting an anthropogenic origin,  
455 while NNV contaminated mussels were detected in both inside and offshore waters of Ravenna's  
456 harbor, mainly during warmer months. These findings reflect the different epidemiological features  
457 of these infections and point out the importance to choose the correct indicator to monitor viral  
458 contaminations.

459 The high frequency of contamination pointed out by this study stresses the imperative to monitor  
460 viral contamination in all coastal habitats where the high natural value meets a number of  
461 recreational and commercial activities such as the Ravenna's harbor area and obtained results  
462 provide the starting point for the development of a more structured bio-monitoring program.

463

#### 464 **Acknowledgements**

465 MP collaborates to the World Harbour Project (<http://www.worldharbourproject.org>), of which  
466 Ravenna is a study case. The authors thank Enrique Riera Ferrer for English language revision of  
467 the manuscript.

468 This research received no specific grant from any funding agency in the public, commercial, or not-  
469 for-profit sectors.

470

#### 471 **Conflict of Interest.**

472 The authors declare that they have no conflict of interest.

473

#### 474 **References**

475 Abbiati, M., Mistri, M., Bartoli, M., Ceccherelli, V.U., Colangelo, M.A. *et al.* (2010). Trade-off  
476 between conservation and exploitation of the transitional water ecosystems of the northern  
477 Adriatic Sea. *Chem Ecol* **26**, 105-119.

478 Airoidi, L. and Beck, M.W. (2007). Loss, status and trends for coastal marine habitats of Europe.  
479 *Oceanogr Mar Biol Annu Rev* **45**, 345-405.

480 Airoidi, L., Ponti, M. and Abbiati, M. (2016). Conservation challenges in human dominated  
481 seascapes: the harbour and coast of Ravenna. *Reg Stud Mar Sci* **8**, 308-318.

482 Airoidi, L., Turon, X., Perkol-Finkel, S. and Rius, M. (2015). Corridors for aliens but not for  
483 natives: effects of marine urban sprawl at a regional scale. *Divers Distrib* **21**, 755-768.

484 Boxman, I.L., Tilburg, J.J., Te Loeke, N.A., Vennema, H., Jonker, K., *et al.* (2006). Detection of  
485 noroviruses in shellfish in the Netherlands. *J Food Microbiol* **108**, 391-396.

486 Burkhardt, W., III, Watkins, W.D. and Rippey, S.R. (1992). Seasonal effects on accumulation of  
487 microbial indicator organisms by *Mercenaria mercenaria*. *Appl Environ Microbiol* **58**, 826-  
488 831.

489 Chironna, M., Grottola, A., Lanave C., Villa, E., Barbuti, S. and Quarto, M. (2003). Genetic  
490 analysis of HAV strains recovered from patients with acute hepatitis from southern Italy. *J*  
491 *Med Virol* **70**, 343-349.

492 Ciulli, S., Gallardi, D., Scagliarini, A., Battilani, M., Hedrick, R.P. and Prosperi, S. (2006).  
493 Temperature-dependency of Betanodavirus infection in SSN-1 cell line. *Dis Aquat Organ* **68**,  
494 261-265.

495 Ciulli, S., Galletti, E., Grodzki, M., Alessi, A., Battilani, M. and Prosperi, S. (2007). Isolation and  
496 genetic characterization of *Betanodavirus* from wild marine fish from the Adriatic Sea. *Vet*  
497 *Res Commun* **31**, 221-224.

498 Ciulli, S., Grodzki, M., Bignami, G., Serratore, P. and Prosperi, S. (2010). Molecular detection and  
499 genetic analysis of *Betanodavirus* in bivalve mollusk. *J Biotechnol* **150S**, S4.

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

503 Croci, L., Losio, M. N., Suffredini, E., Pavoni, E., Di Pasquale, S. (2007). Assessment of human  
504 enteric viruses in shellfish from the northern Adriatic Sea. *International J Food Microbiol*  
505 **114**, 252-257.

506 Da Silva, A.K., Le Saux, J.C., Parnaudeau, S., Pommepuy, M., Elimelech, M. and Le Guyader,  
507 F.S. (2007). Evaluation of removal of noroviruses during wastewater treatment using real-  
508 time reverse transcription-PCR: Different behaviors of genogroups I and II. *Appl Environ*  
509 *Microbiol* **73**, 7891-7897.

510 Doan, Q. K., Vandeputte, M., Chatain, B., Morin, T. and Allal, F. (2017). Viral encephalopathy  
511 and retinopathy in aquaculture: a review. *J Fish Dis* **40**, 717-742.

512 Drake, L., Doblin, M.A. and Dobbs, F.C. (2007). Potential microbial bioinvasions via ships' ballast  
513 water, sediment, and biofilm. *Mar Pollut Bull* **55**, 333-341.

514 Fabi, G., Grati, F., Puletti, M. and Scarcella, G. (2004). Effects on fish community induced by  
515 installation of two gas platforms in the Adriatic Sea. *Mar Ecol Progr Ser* **273**, 187-197.

516 Goldbergh, E.D., Bowen, V.T., Farrington, J.W., Harvey, G., Martin, J.H. *et al.* (1978). The mussel  
517 watch. *Environ Conserv* **5**, 101-125.

518 Gonçalves, J., Gutiérrez-Aguirre, I., Balasubramanian, M.N., Zagorščak, M., Ravnikar, M. and  
519 Turk, V. (2018). Surveillance of human enteric viruses in coastal waters using concentration  
520 with methacrylate monolithic supports prior to detection by RT-qPCR. *Mar Pollut Bull* **128**,  
521 307-317.

522 Green, J., Henshilwood, K., Gallimore, C.I., Brown, D.W. and Lees, D.N. (1998). A Nested  
523 Reverse Transcriptase PCR assay for detection of small round-structured viruses in  
524 environmentally contaminated molluscan shellfish. *Appl Environ Microbiol* **64**, 858-863.

525 Henigman, U., Biasizzo, M., Vadnjal, S., Toplak, I., Gombac, M. *et al.* (2015). Molecular  
526 characterization of noroviruses detected in mussels (*Mytilus galloprovincialis*) from  
527 harvesting areas in Slovenia. *New Microbiol* **38**, 225-233.

528 Ilic, N., Velebit, B., Teodorovic, V., Djordjevic, V., Karabasil, N. *et al.* (2017). Influence of  
529 environmental conditions on norovirus presence in mussels harvested in Montenegro. *Food*  
530 *Environ Virol* **9**, 406-414.

531 Kageyama, T., Kojima, S., Shinohara, M., Uchida, K., Fukushi, S. *et al.* (2003). Broadly reactive  
532 and highly sensitive assay for Norwalk-like viruses based on real-time quantitative reverse  
533 transcription-PCR. *J Clin Microbiol* **41**, 1548-1557.

534 Kim, Y., Aw, T.G. and Rose, J.B. (2016). Transporting oceanic viromes: Invasion of the aquatic  
535 biosphere. *Plos One* **11**, e0152671.

536 Kim, Y.C., Kwon, W.J., Kim, M.S., Kim, K.I., Min, J.G. and Jeong, H.D. (2018). High prevalence  
537 of betanodavirus barfin flounder nervous necrosis virus as well as red-spotted grouper  
538 nervous necrosis virus genotype in shellfish. *J Fish Dis* **41**, 233-246.

539 Kroneman, A., Vennema, H., Deforche, K., Avoort, H.V., Peñarada, S. *et al.* (2011). An automated  
540 genotyping tool for enteroviruses and noroviruses. *J Clin Virol* **51**, 121-125.

541 La Bella, G., Martella, V., Basanisi, M.G., Nobili, G., Terio, V. and La Salandra, G. (2017). Food-  
542 borne viruses in shellfish: Investigation on norovirus and HAV presence in Apulia (SE Italy).  
543 *Food Environ Virol* **9**, 179-186.

544 Le Guyader, F., Dubois, E., Menard, D. and Pommepuy, M. (1994). Detection of hepatitis A virus,  
545 rotavirus, and enterovirus in naturally contaminated shellfish and sediment by reverse  
546 transcription-semi-nested PCR. *Appl Environ Microbiol* **60**, 3665-3671.

547 Lipp, E.K., Kurz, R., Vincent, R. and Rodriguez-Palacios, C. (2001). The effects of seasonal  
548 variability and weather on microbial fecal pollution and enteric pathogens in a subtropical  
549 estuary. *Estuaries* **24**, 266-276.

550 Loisy, F., Atmar, R.L., Gullon, P., Le Cann, P., Pommepuy, M. and Le Guyader, F.S. (2005). Real-  
551 time RT-PCR for norovirus screening in shellfish. *J Virol Methods* **123**, 1-7.

552 Maalouf, H., Pommepuy, M. and Le Guyader, F. S. (2010). Environmental conditions leading to  
553 shellfish contamination and related outbreaks. *Food Environ Virol* **2**, 136-145.

554 Markich, S.J. and Jeffree, R.A. (2019). The euryhaline pygmy mussel, *Xenostrobus securis*, is a  
555 useful biomonitor of key metal contamination in the highly urbanised Sydney Estuary,  
556 Australia. *Environ Pollut* **252**, 813-824.

557 Mbayed, V. A., Sookoian, S., Alfonso, V. and Campos, R. H. (2002). Genetic characterization of  
558 hepatitis A virus isolates from Buenos Aires, Argentina. *J Med Virol* **68**, 168-174.

559 Medici, M.C., Tummolo, F., Martella, V., Chezzi, C., Arcangeletti, M.C. *et al.* (2014).  
560 Epidemiological and molecular features of norovirus infections in Italian children affected  
561 with acute gastroenteritis. *Epidemiol Infect* **142**, 2326-2335.

562 Middleboe, M. and Brussaard, C. P.D. (2017). Marine viruses: Key players in marine ecosystems.  
563 *Viruses* **9**, 302.

564 Molloy, S.D., Pietrak, M.R., Bricknell, I. and Bouchard, D.A. (2013). Experimental transmission of  
565 infectious pancreatic necrosis virus from the blue mussel, *Mytilus edulis*, to cohabitating  
566 Atlantic salmon (*Salmo salar*) smolts. *Appl Env Microbiol* **79**, 5882-5890.

567 Munn, C. (2006). Viruses as pathogens of marine organisms- from bacteria to whales. *J Mar Biol*  
568 *Ass UK* **86**, 453-467.

569 Nishizawa, T., Mori, K., Nakai, T., Furusawa, I. and Muroga, K. (1994). Polymerase chain reaction  
570 (PCR) amplification of RNA of striped jack nervous necrosis virus (SJNNV). *Dis Aquat*  
571 *Organ* **18**, 103-107.

572 Occhipinti-Ambrogi, A. (2000). Biotic invasions in a Mediterranean Lagoon. *Biol Invasions* **2**,  
573 165-176.

574 Oliveira, J. G., Souto, S., Dopazo, C. P., Thiéry, R., Barja, J. L., and Bandín, I. (2009). Comparative  
575 analysis of both genomic segments of betanodaviruses isolated from epizootic outbreaks in  
576 farmed fish species provides evidence for genetic reassortment. *J Gen Virol* **90**, 2940-2951.

577 Panzarin, V., Fusaro, A., Monne, I., Cappellozza, E., Patarnello, P. *et al.* (2012). Molecular  
578 epidemiology and evolutionary dynamics of betanodavirus in southern Europe. *Infect, Genet*  
579 *Evol* **12**, 53-61.

580 Ponti, M., Abbiati, M. and Ceccherelli, V.U. (2002). Drilling platforms as artificial reefs:  
581 distribution of microbenthic assemblages of the “Paguro” wreck (northern Adriatic Sea).  
582 *ICES J Mar Sci* **59**, S316-S323.

583 Ponti, M., Colangelo, M.A. and Ceccherelli, V.U. (2007). Composition, biomass and secondary  
584 production of the macrobenthic invertebrate assemblages in a coastal lagoon exploited for  
585 extensive aquaculture: Valle Smarlacca (northern Adriatic Sea). *Estuar Coast Shelf S* **75**, 79-  
586 89.

587 Ponti, M., Pasteris, A., Guerra, R. and Abbiati, M. (2009). Impacts of maintenance channel  
588 dredging in a northern Adriatic coastal lagoon. II: Effects on macrobenthic assemblages in  
589 channels and ponds. *Estuar Coast Shelf S* **85**, 143-150.

590 Ponti, M., Casselli, C. and Abbiati, M. (2011). Anthropogenic disturbance and spatial  
591 heterogeneity of macrobenthic invertebrate assemblages in coastal lagoons: the study case of  
592 Pialassa Baiona (northern Adriatic Sea). *Helgol Mar Res* **65**, 25-42.

593 Ponti, M., Castellini, A., Ragazzoni, A., Gamba, E., Ceccherelli, V.U. and Abbiati, M. (2017).  
594 Decline of the Manila clams stock in the northern Adriatic lagoons: a survey on ecological  
595 and socio-economic aspects. *Acta Adriat* **58**, 89-104.

596 Pranovi, F., Colla, S., Valeri, P. and Anelli Monti, M. (2016). Present and future status of artisanal  
597 fisheries in the Adriatic Sea (western Mediterranean Sea). *Ocean Coast Manage* **122**, 49-56.

598 Serratore, P., Ciulli, S., Piano, A. and Cariani, A. (2014). Criticism of the purification process of  
599 bivalve shellfish: Literature review and our industrial research experiences. In *Shellfish*  
600 *human consumption, health implications and conservation concerns*, Ed. Hay R.M. Nova  
601 Science Publishers, Inc., New York, USA. pp. 1-50.

602 Svraka, S., Duizer, E., Vennema, H., de Bruin, E., van der Veer, B. *et al.* (2007). Etiological role of  
603 viruses in outbreaks of acute gastroenteritis in the Netherlands from 1994 through 2005. *J*  
604 *Clin Microbiol* **45**, 1389-1394.

605 Suffredini, E., Corrain, C., Arcangeli, G., Fasolato, L., Manfrin, A. *et al.* (2008). Occurrence of  
606 enteric viruses in shellfish and relation to climatic-environmental factors. *Lett Appl Microbiol*  
607 **47**, 467-74.

608 Suffredini, E., Magnabosco, C., Civettini, M., Rossetti, E., Arcangeli, G. and Croci, L. (2012).  
609 Norovirus contamination in different shellfish species harvested in the same production areas.  
610 *J Appl Microbiol* **113**, 686-692.

611 Toffolo, V., Negrisolo, E., Maltese, C., Bovo, G., Belvedere, P. *et al.* (2007). Phylogeny of  
612 betanodaviruses and molecular evolution of their RNA polymerase and coat proteins. *Mol*  
613 *Phylogenet Evol* **43**, 298-308.

614 van Beek, J., de Graaf, M., Al-Hello, H., Allen, D.J., Ambert-Balay, K. *et al.* (2018). Molecular  
615 surveillance of norovirus, 2005-16: an epidemiological analysis of data collected from the  
616 NoroNet network. *The Lancet Infect Dis* **18**, 545-553.

617 Vendramin, N., Patarnello, P., Toffan, A., Panzarin, V., Cappelozza, E. *et al.* (2013). Viral  
618 Encephalopathy and Retinopathy in groupers (*Epinephelus* spp.) in southern Italy: a threat for  
619 wild endangered species. *BMC Vet Res* **9**, 20.

620 Verhoef, L., Hewitt, J., Barclay, L., Ahmed, S.M., Lake, B. *et al.* (2015). Norovirus genotype  
621 profiles associated with foodborne transmission, 1999-2012. *Emerg Infect Dis* **21**, 592-599.

622 Vinjé, J. and Koopmans, M.P.G. (1996). Molecular Detection and Epidemiology of Small Round-  
623 Structured Viruses in Outbreaks of Gastroenteritis in the Netherlands. *J Infect Dis* **174**, 610-  
624 615.

625 Volpe, E., Pagnini, N., Serratore, P. and Ciulli, S. (2017). Fate of redspottet grouper nervous  
626 necrosis virus (RGNNV) in experimentally challenged Manila clam *Ruditapes*  
627 *philippinarum*. *Dis Aquat Organ* **125**, 53-61.

628 Volpe, E., Grodzki, M., Panzarin, V., Guercio, A., Purpari, G. *et al.* (2018). Detection and  
629 molecular characterization of betanodaviruses retrieved from bivalve molluscs. *J Fish Dis*,  
630 **41**, 603-611.

631 Volpe, E., Gustinelli, A., Caffara, M., Errani, F., Quaglio, F. *et al.* (2020). Viral nervous necrosis  
632 outbreaks caused by the RGNNV/SJNNV reassortant betanodavirus in gilthead sea bream  
633 (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*). *Aquaculture* **523**, 735155.  
634 doi: 10.1016/j.aquaculture.2020.735155

635 Wang, Y., Zhang, J. and Shen, Z. (2015). The impact of calicivirus mixed infection in an oyster-  
636 associated outbreak during a food festival. *J Clin Virol*, **73**, 55-63.

637 Wyn-Jones P.A., Carducci, A., Cook N., D'Agostino, M., Divizia, M. *et al.* (2011). Surveillance of  
638 adenoviruses and noroviruses in European recreational waters. *Water Res* **45**, 1025-1038.

**Table 1.** Details of primers and probes used in this study.

Target virus	Name	Sequence (5'→3')	Target region	Reference
<b>HAV RT nested-PCR</b>	AV1	5'-GGAAATGTCTCAGGTACTTTCTTTG-3'	<b>VP1</b>	Le Guyader et al. 1994
	AV2	5'-GTTTTGCTCCTCTTTATCATGCTATG-3'		
	AV3	5'-TCCTCAATTGTTGTGATAGC-3'		
<b>NoV Real time PCR</b>	QNIF4	5'-CGTGGATGCGNTTCCAT-3'	<b>RdRp</b>	Da Silva et al. 2007
	NV1LCR	5'-CCTTAGACGCCATCATCATTTAC-3'		Svraka et al. 2007
	NVGG1p	5'-FAM-TGGACAGGAGAYCGCRATCT-3'TAMRA		Loisy et al. 2005
	QNIF2	5'-ATGTTCAAGRTGGATGAGRTTCTCWGA-3'		Kageyama et al. 2003
	COG2R	5'-TCGACGCCATCTTCATTCACA-3'		Loisy et al. 2005
	QNIFS	5'-FAM-AGCACGTGGGAGGGCGATCG-3'TAMRA		
<b>NoV RT-nested PCR</b>	JV12	5'-ATACCACTATGATGCAGATTA-3'	<b>RdRp</b>	Vinjé and Koopmans, 1996
	JV13	5'-TCATCATCACCATAGAAAGAG-3'		
	NVG1	5'-TCNGAAATGGATGTTGG-3'		Green et al. 1998
	NVG2	5'-AGCCAGTGGGCGATGGAATTC-3'		Boxman et al. 2006
<b>NNV RT nested-PCR</b>	VNNV5	5'-GTTGAGGATTATCGCCAACG-3'	<b>RNA1</b>	Toffolo et al. 2007
	VNNV6	5'-ACCGGCGAACAGTATCTGAC-3'		
	VNNV7	5'-CACTACCGTGTTGCTG-3'		
<b>NNV RT nested-PCR</b>	S6	5'-ATGGTACGCAAAGGTGATAAGAAA-3'	<b>RNA2</b>	Ciulli et al. 2006
	S7	5'-GTTTTCCGAGTCAACACGGGT-3'		
	F2	5'-CGTGTCAGTCATGTGTCGCT-3'		Nishizawa et al. 1994
	R3	5'-CGAGTCAACACGGGTGAAGA-3'		

643 **Table 2.** Presence of human viruses in the sampled sites.

644

645

646

647

648

649

<b>Year</b>	<b>2018</b>				<b>2019</b>		
<b>Month</b>	<b>March</b>	<b>May</b>	<b>July</b>	<b>September</b>	<b>November</b>	<b>January</b>	<b>March</b>
<b>St. 1</b>	n.d.	Negative	Negative	Negative	Negative	Negative	Negative
<b>St. 2</b>	<b>NoV</b>	Negative	Negative	Negative	Negative	Negative	<b>NoV</b>
<b>St. 5</b>	<b>NoV, HAV</b>	<b>NoV</b>	Negative	Negative	Negative	<b>NoV</b>	<b>NoV</b>
<b>St. 6</b>	n.d.	<b>NoV, HAV</b>	Negative	Negative	<b>NoV</b>	<b>NoV</b>	<b>NoV</b>
<b>Platform GAR A/C</b>	n.d.	n.d.	Negative	Negative	n.d.	n.d.	n.d.
<b>Platform ANTARES/AMELIA</b>	n.d.	n.d.	Negative	Negative	n.d.	n.d.	n.d.

650

651 n.d. not determined

652

653

654

655

656

657

658

659

660

661 **Table 3.** Presence of NNV in sampled sites

662

663

664

665

666

667

668

<b>Year</b>	<b>2018</b>				<b>2019</b>		
<b>Month</b>	<b>March</b>	<b>May</b>	<b>July</b>	<b>September</b>	<b>November</b>	<b>January</b>	<b>March</b>
<b>St. 1</b>	n.d.	Negative	<b>Positive</b>	<b>Positive</b>	Negative	Negative	Negative
<b>St. 2</b>	<b>Positive</b>	Negative	<b>Positive</b>	<b>Positive</b>	Negative	Negative	Negative
<b>St. 5</b>	<b>Positive</b>	<b>Positive</b>	Negative	Negative	Negative	Negative	Negative
<b>St. 6</b>	n.d.	<b>Positive</b>	<b>Positive</b>	Negative	Negative	Negative	Negative
<b>Platform GAR A/C</b>	n.d.	n.d.	Negative	<b>Positive</b>	n.d.	n.d.	n.d.
<b>Platform ANATARES/AMELIA</b>	n.d.	n.d.	Negative	<b>Positive</b>	n.d.	n.d.	n.d.

669 n.d. not determined

670

671

672

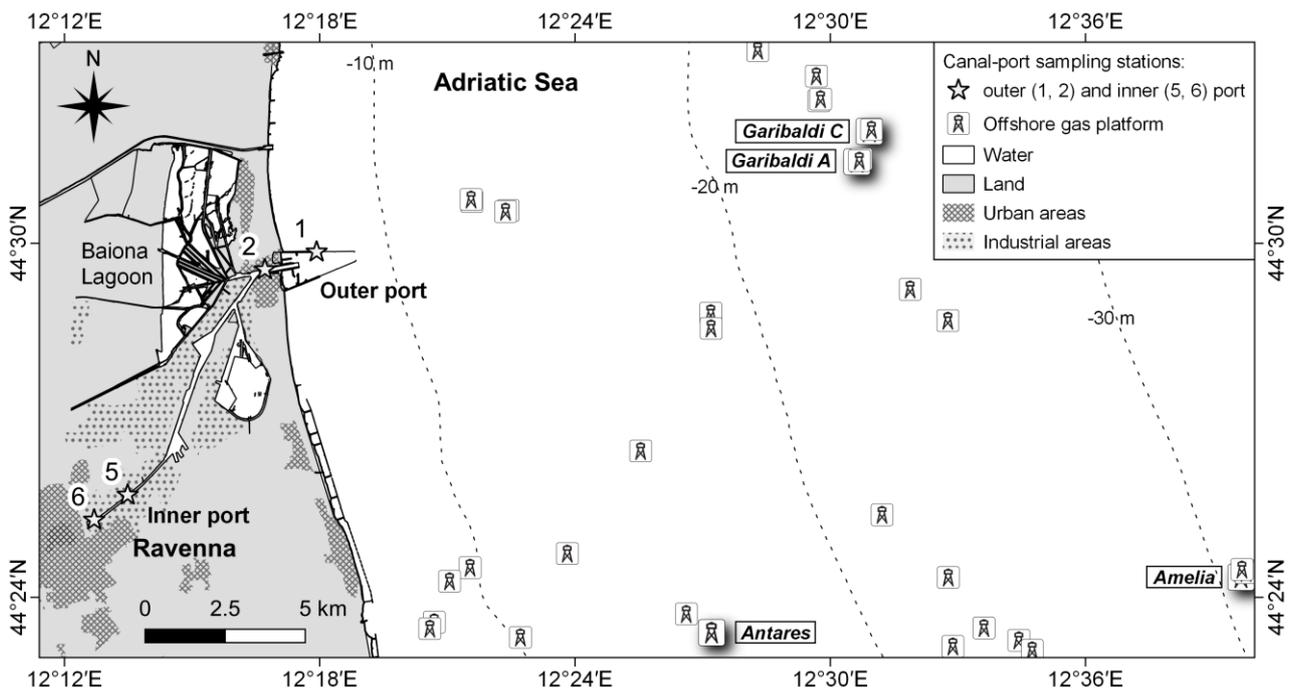
673

674

675

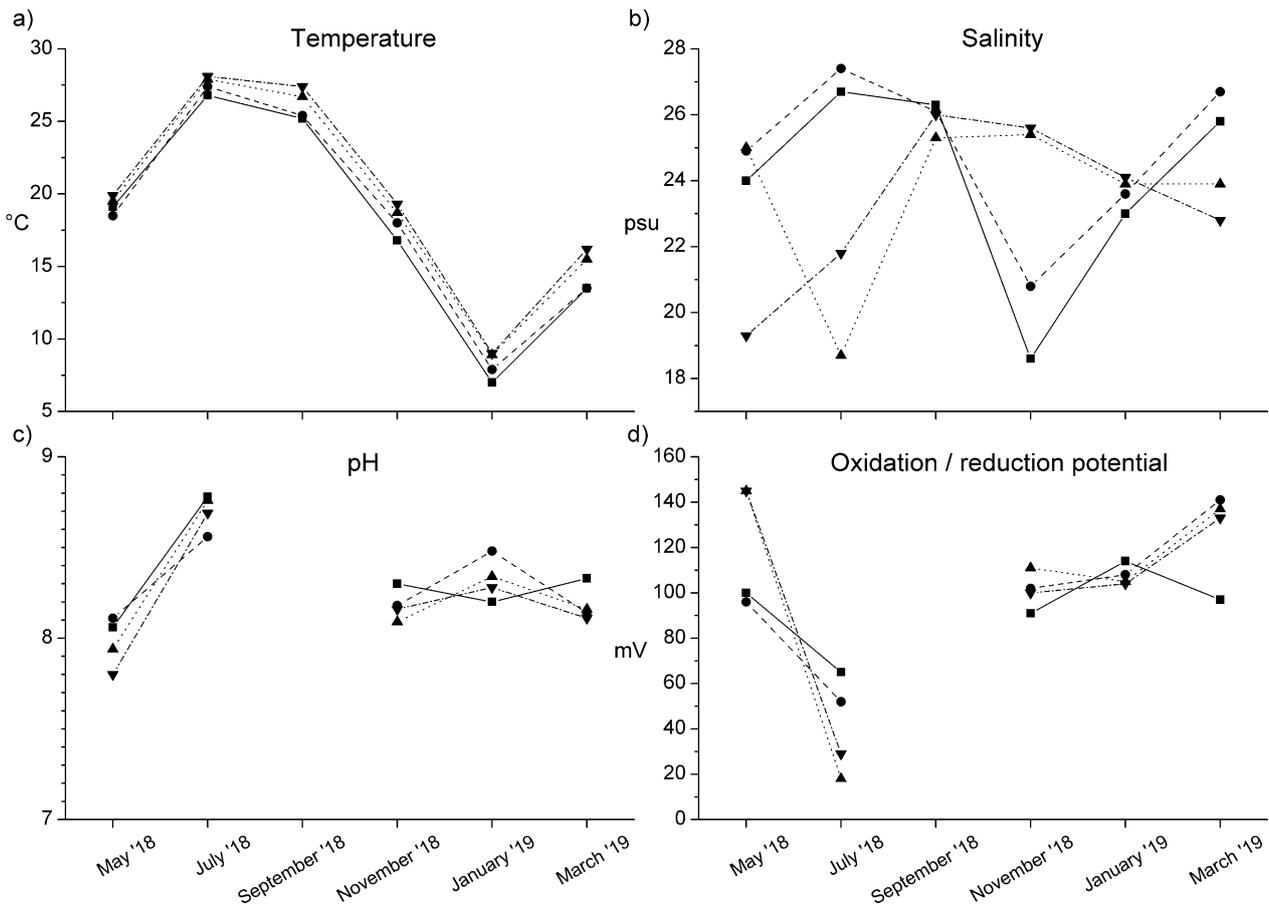
676

677 **Figure legends**



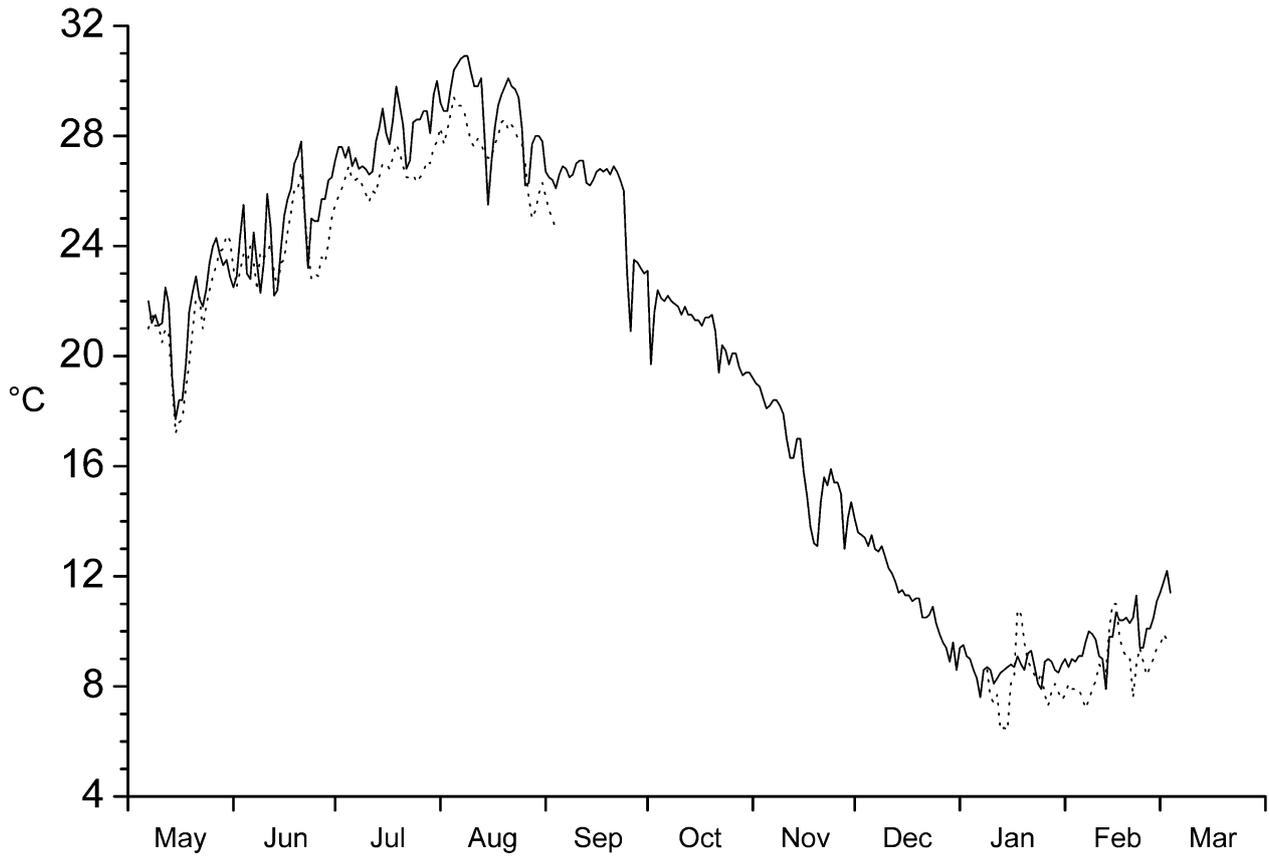
678

679 **Fig. 1** Map showing the sampling sites within the Ravenna's canal port (☆) labelled as: St. 1 and  
680 St. 2 in the outer port and St. 5 and St. 6 in the inner port; and at offshore gas platforms (⊞) labelled  
681 as: Garibaldi A and C, Amelia and Antares. Geographic coordinates in WGS84, Mercator  
682 projection.



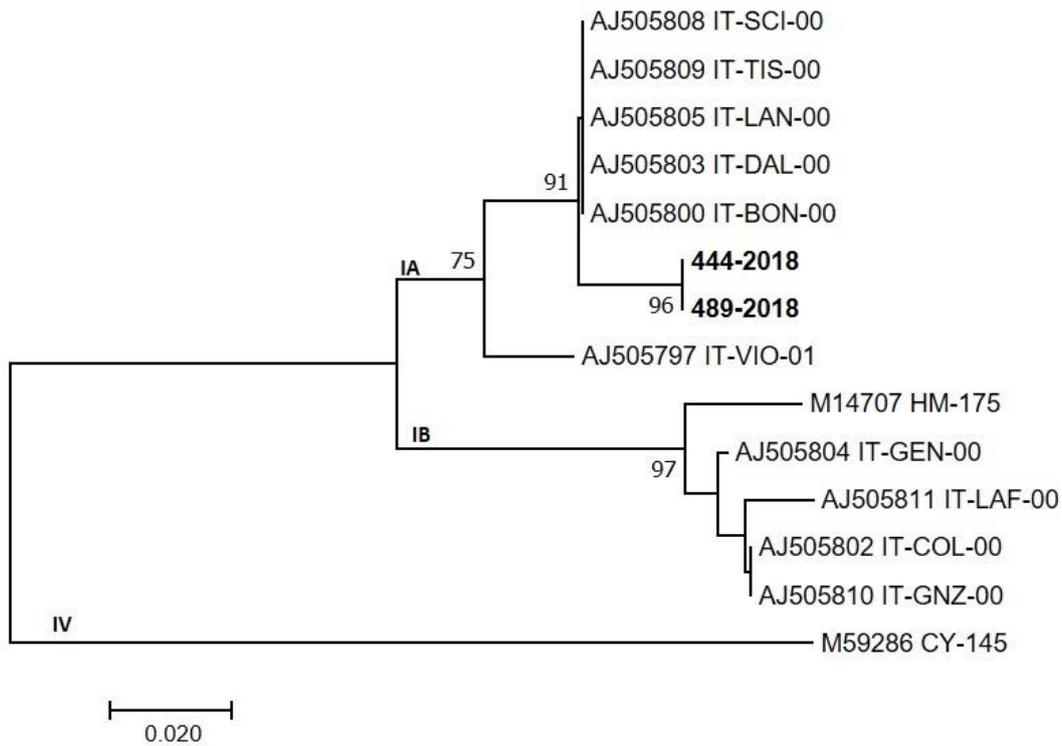
683

684 **Fig. 2** Water temperature (a), salinity (b), pH (c) and oxidation / reduction potential (d) measured at  
 685 mussel sampling times within the Ravenna's canal port. Outer port: St. 1 (■) and St. 2 (●); inner  
 686 port: St. 5 (▲) and St. 6 (▼).



687

688 **Fig. 3** Mean daily water temperature at the outer port (St. 1, dotted line) and at the inner port (St. 6,  
 689 solid line) from May 2018 to March 2019. Measurements at St. 1 were interrupted due to the losses  
 690 of the probe caused by an exceptional storm occurred in November 2018.



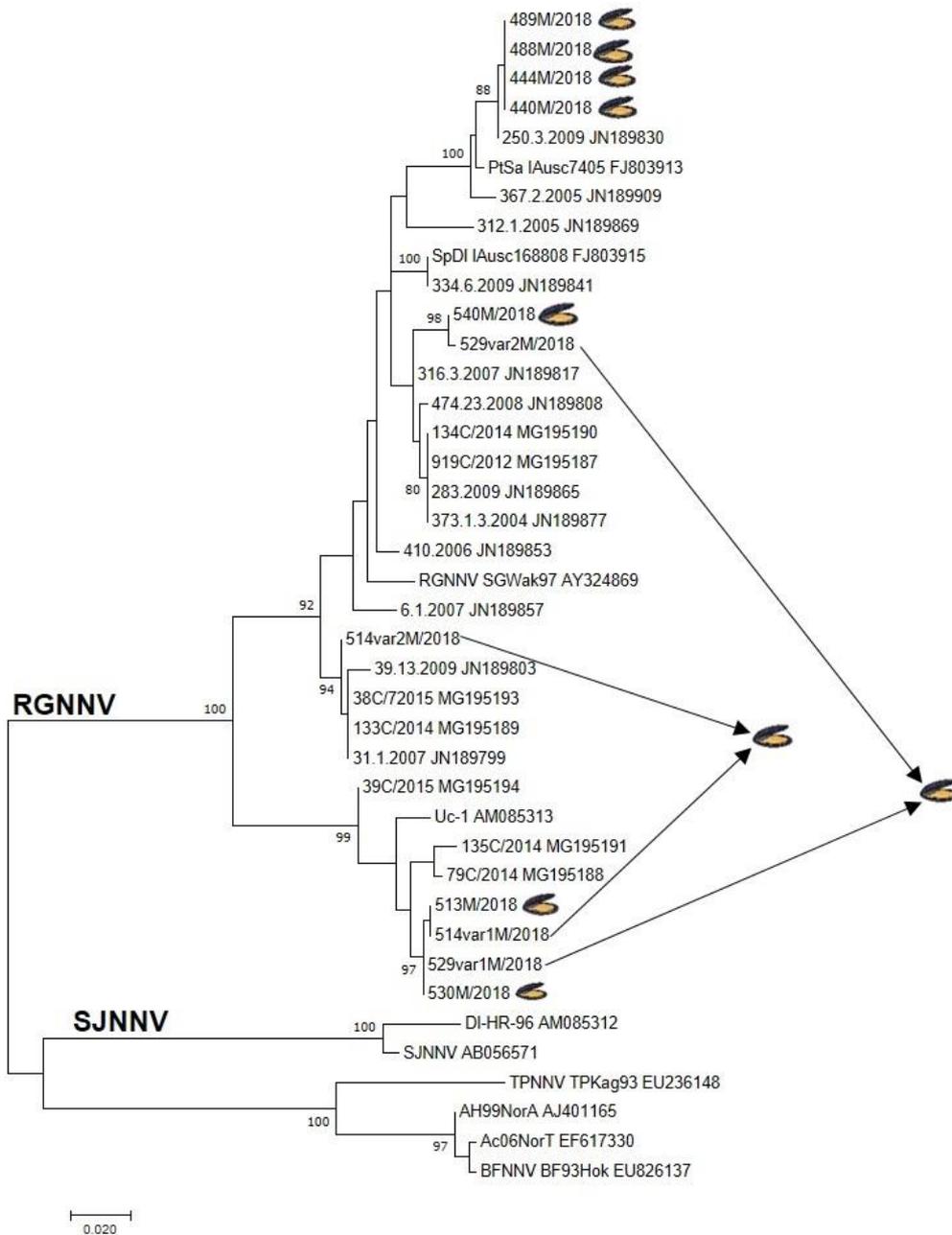
691

692 **Fig. 4** Neighbor-joining phylogenetic tree based on the partial VP1 nucleotide sequences (185 bp).

693 Sequences retrieved from GenBank are reported with the isolate name and accession number.

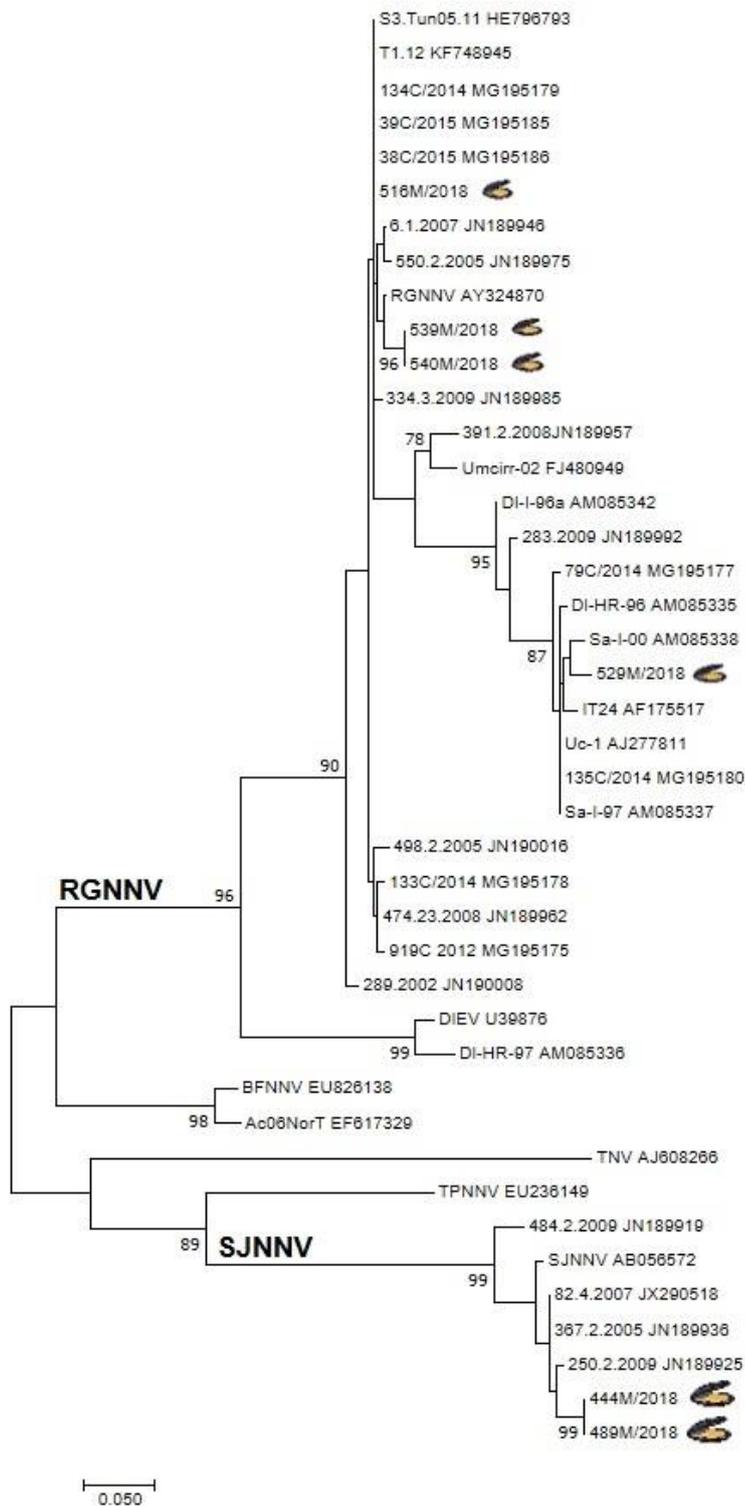
694 Bootstrap values >70% are shown. Branch lengths are scaled according to the number of nucleotide

695 substitutions per site. The scale bar is reported.



696

697 **Fig. 5** Maximum likelihood phylogenetic tree based on partial RNA1 nucleotide sequences (419  
 698 bp). Sequences retrieved from GenBank are reported with the isolate name and accession number.  
 699 Bootstrap values >70% are shown. Branch lengths are scaled according to the number of nucleotide  
 700 substitutions per site. The scale bar is reported.



701

702 **Fig. 6** Maximum likelihood phylogenetic tree based on partial RNA2 nucleotide sequences (281

703 bp). Sequences retrieved from GenBank are reported with the isolate name and accession number.

704 Bootstrap values >70% are shown. Branch lengths are scaled according to the number of nucleotide

705 substitutions per site. The scale bar is reported.

706

707 **Supporting Information**

708 **Table S1:** Physical-chemical water parameters collected within the Ravenna's canal port at mussel

709 sampling times.

710