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

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Abstract

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

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

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

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

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

Introduction

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

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

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

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

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

The aim of this study was to investigate the presence of environmental contamination by human and fish viruses inside and offshore of Ravenna's harbor monitoring mussels as bio-vectors. Particularly, the presence of human viruses like NoV and HAV and the presence of one of the most threatening finfish pathogens, the NNV, was investigated considering their spatial and seasonal

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

Materials and method

Study area

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

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

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

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

Sampling design and field activities

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

The sampling design aimed to investigate three areas with different environmental features: the 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), 1.2-2.8 km from the port entrance, and the offshore area (gas platforms; Fig. 1).

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

[1] 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

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

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

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

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

[2] http://blast.ncbi.nlm.nih.gov/Blast.cgi
[3] www.ncbi.nlm.nih.gov

using Clustal W implemented in the BioEdit software[4]. Neighbor-joining phylogenetic analysis of the partial VP1 gene was performed with MEGA 7 software[5]. Bootstrap analysis was carried out on 1,000 replicates.

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

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

Data availability

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

Statistical analyses

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

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

[4] http://bioedit.software.informer.com/
[5] www.megasoftware.net

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

Results

Physical-chemical parameters

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

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

Human viruses' detection and genotyping

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

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

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

A VP1 gene fragment was sequenced for HAV detected in mussels sampled in March 2018 and May 2018. The two sequences showed a 100% nucleotide identity. The detected HAV strain exhibited the highest nucleotide identity (98-99%) with sequences of HAV subtype IA (Genbank accession numbers: MF416223; AY441441; AJ505803; AJ505800). Phylogenetic analysis of HAV 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

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

Discussion

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

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

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

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

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

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

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

Also for NoV, the genogroup and genotype determination have been useful to monitor the global spread of noroviruses (Henigman et al. 2015; van Beek et al. 2018). Furthermore, genotype profiles may help to distinguish the origin of the outbreaks; the NoV genogroup I (GI) represents the most frequently implicated in shellfish and water-related outbreaks (Maalouf et al. 2010). Conversely, 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 (Croci 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

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

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

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

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

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

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

directly connected to the port, we hypothesized that the NNV could be released directly by infected wild marine finfish species. NNV, in fact, has been previously isolated from several asymptomatic wild marine finfish species (Ciulli et al. 2007). Furthermore, experimental trials have demonstrated that asymptomatic finfish can transmit the infection to susceptible host (Doan et al. 2017). The frequent presence of NNV shown in this study in mussels from inside and offshore waters of Ravenna's harbor suggests that susceptible native finfish host species could be infected and release the virus in the water at high titers during summer. This hypothesis seems to be reinforced by the observation that most of the NNV contaminated mussels have been detected during the July and September sampling. A previous survey on NNV shellfish contamination showed a higher detection rate of RGNNV in summer than in winter, which was similar to the known seasonal patterns of finfish infection (Kim et al. 2018). Furthermore, experimental trials demonstrated that some bivalve mollusk species are able to accumulate and release viable viruses, including NNV (Molloy et al. 2013; Volpe et al. 2017; Kim et al. 2018). The accumulation and release of viable NNV by mussels in Ravenna's harbor area could represent a viral source for other wild susceptible finfish hosts, enabling to complete the epidemiological cycle of NNV infection in the natural environment.

To sum up, virological investigation in inside and offshore waters of Ravenna's harbor showed the presence of several viral contaminations in mussels. Despite the study applied a qualitative methodology without quantifying viral loads, it permitted to successfully detect human and fish virus contaminations in mussels and to evaluate their frequency and distribution. A different spatial and **s**easonal distribution were observed in human and fish virus contaminations. Human viruses were detected mainly in inner port sites during colder months suggesting an anthropogenic origin, while NNV contaminated mussels were detected in both inside and offshore waters of Ravenna's harbor, mainly during warmer months. These findings reflect the different epidemiological features of these infections and point out the importance to choose the correct indicator to monitor viral contaminations.

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

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Conflict of Interest.

The authors declare that they have no conflict of interest.

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

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

640

641

642

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

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

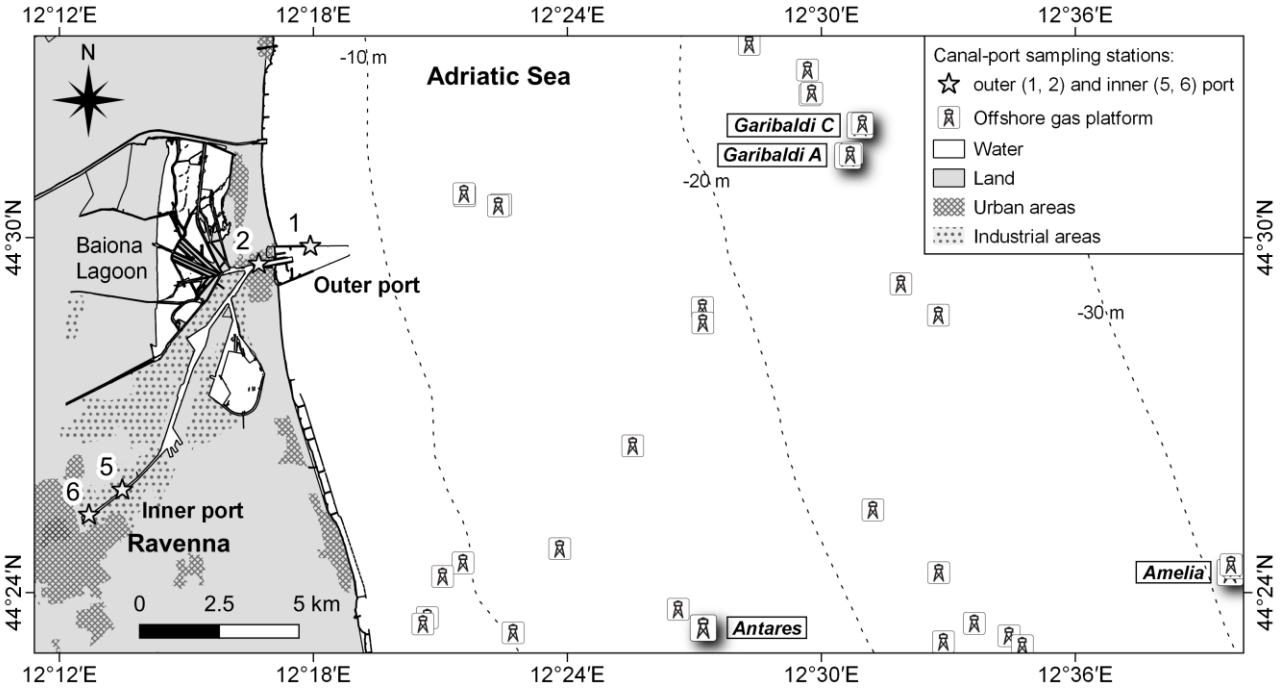
n.d. not determined

Table 3. Presence of NNV in sampled sites

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

n.d. not determined

677 **Figure legends**



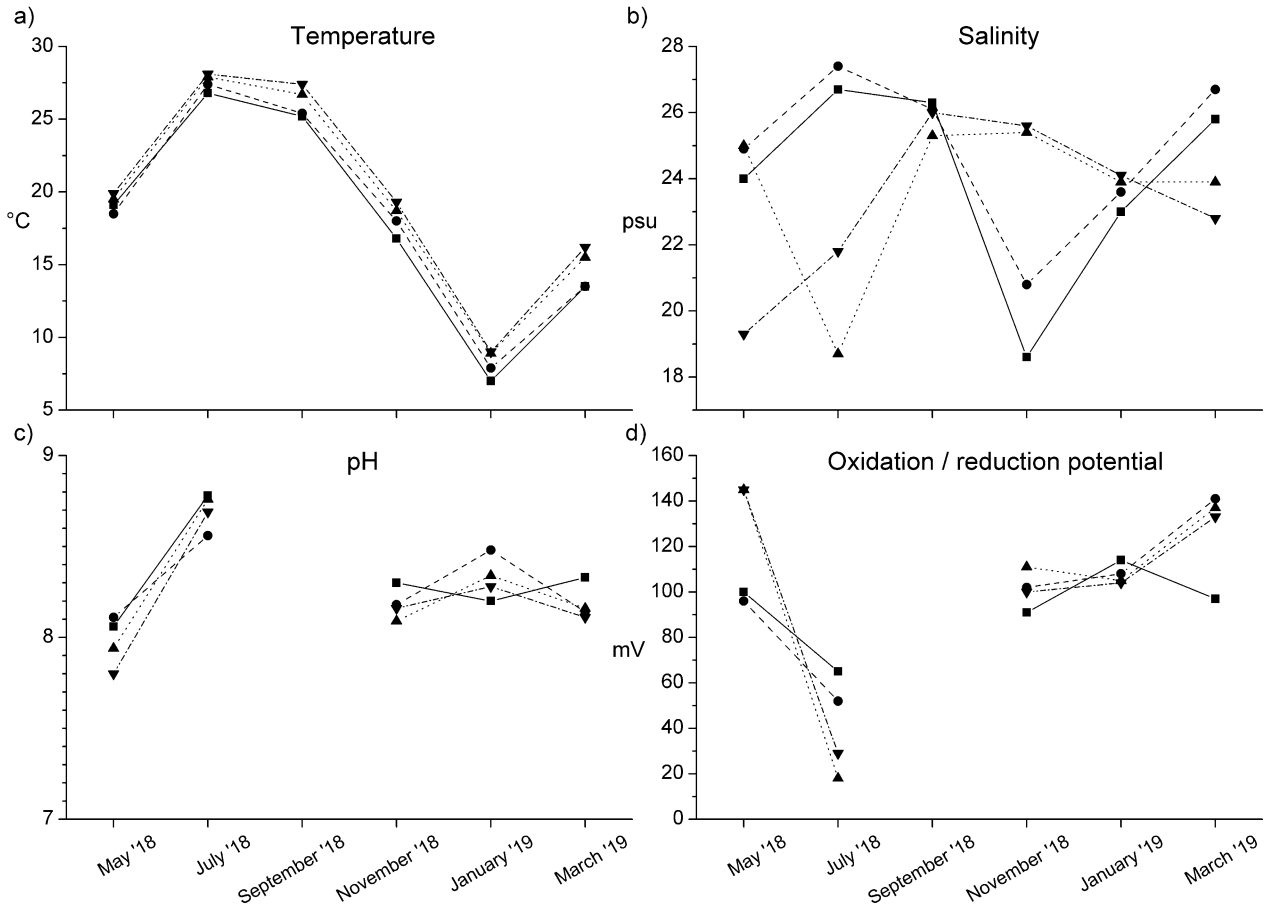
678

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.

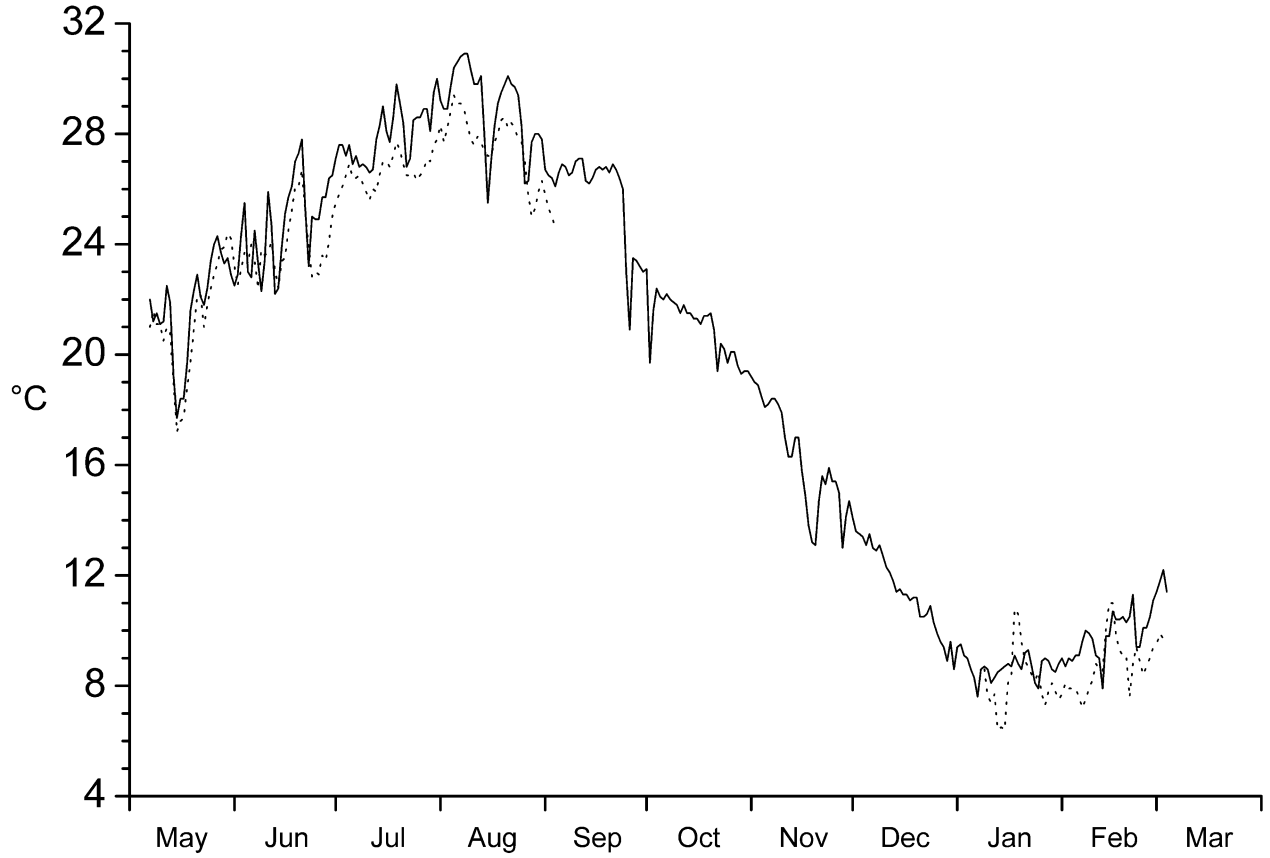


683

684 **Fig. 2** Water temperature (a), salinity (b), pH (c) and oxidation / reduction potential (d) measured at

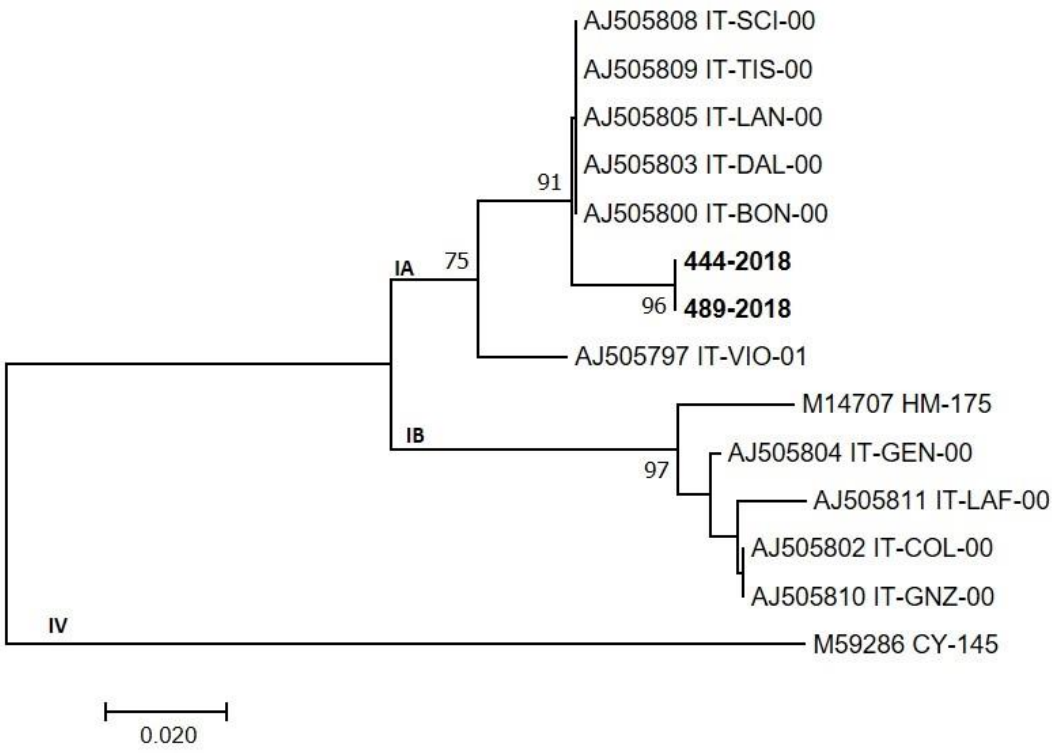
685 mussel sampling times within the Ravenna's canal port. Outer port: St. 1 (■) and St. 2 (●); inner

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.

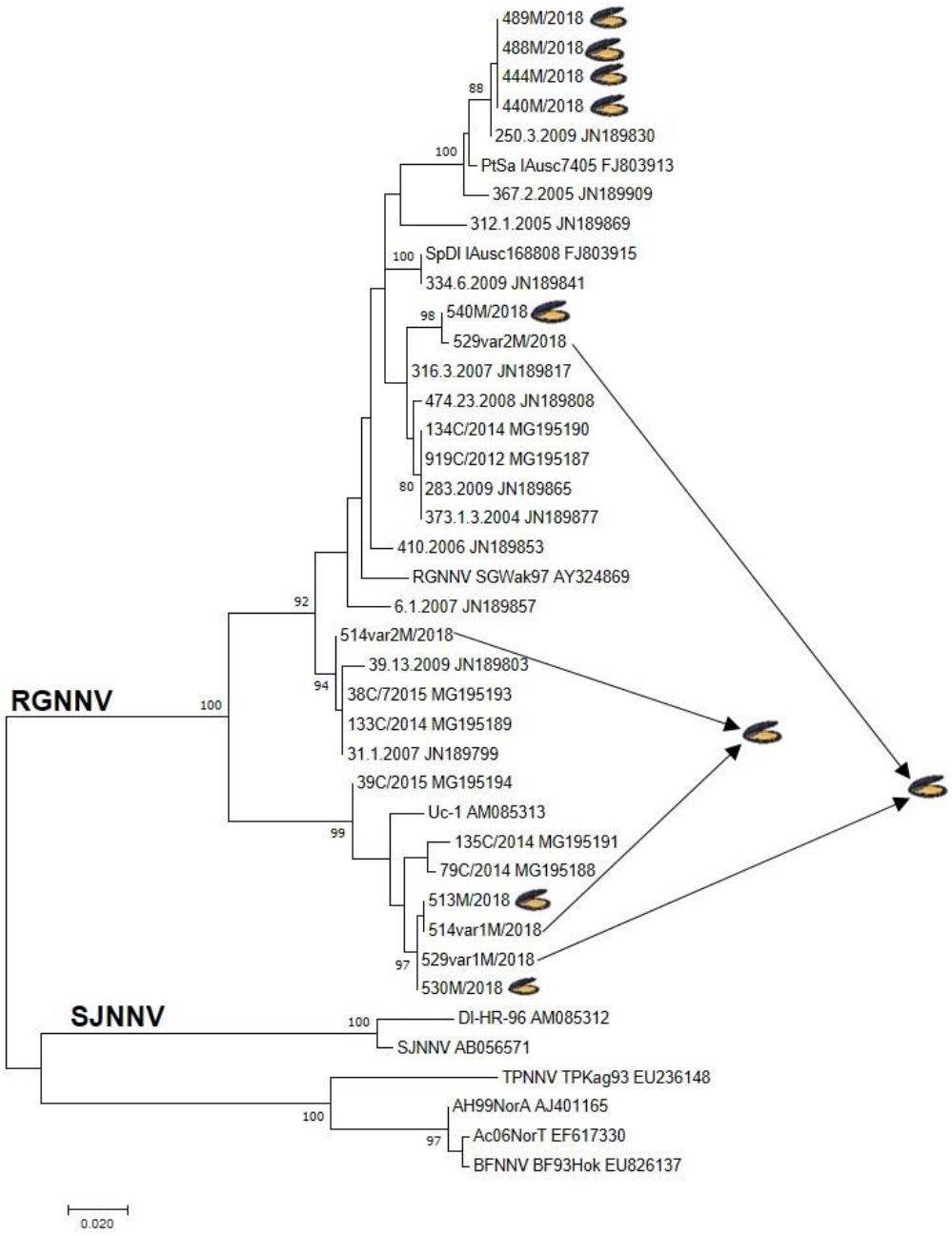
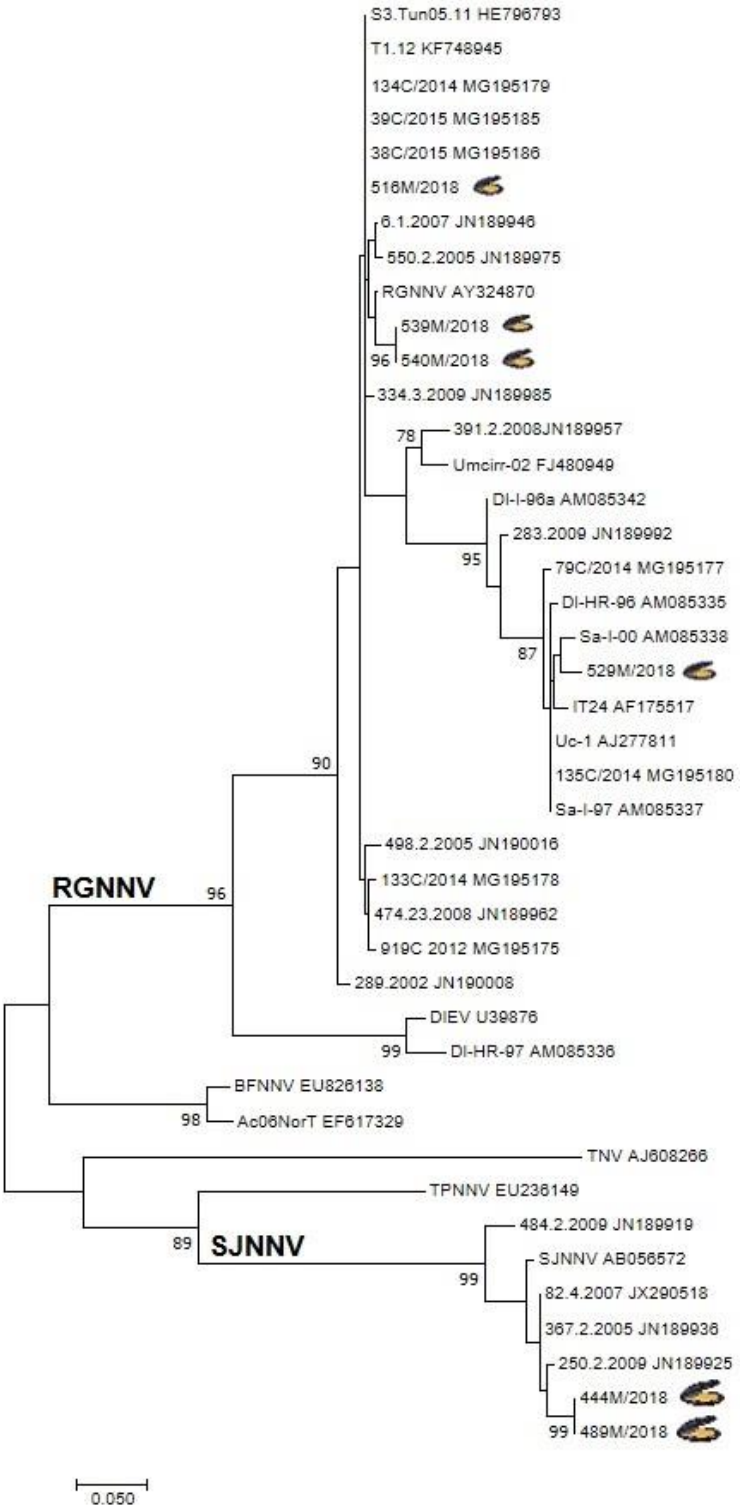


Fig. 5 Maximum likelihood phylogenetic tree based on partial RNA1 nucleotide sequences (419 bp). Sequences retrieved from GenBank are reported with the isolate name and accession number. Bootstrap values >70% are shown. Branch lengths are scaled according to the number of nucleotide substitutions per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

Supporting Information

Table S1: Physical-chemical water parameters collected within the Ravenna's canal port at mussel sampling times.