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Viral investigation in the Ravenna's harbor: mussels as bio-indicators for the presence of Norovirus, Hepatitis A virus and Nervous Necrosis Virus

Relatore:

Dott.ssa Sara Ciulli

Presentata da:

Francesca Errani

Correlatore:

Dr. Massimo Ponti

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Abstract

Ravenna is the major coastal city of Emilia Romagna and one of the largest commercial seaports in Italy. The harbor is structured as a major canal directly connected with two lagoons and, overall, this is an area of renowned environment, cultural and economic value. Human pressure on this area has been historically intense due to urbanization and ship circulation, this leads to many environmental issues: loss of biodiversity, introduction of alien species, loss of habitat and pollution. Species diversity in this environment is relatively high and includes bivalve mollusks that have been proposed as being useful for biomonitoring trace levels of environmental contaminants due to their wide range distribution, sessile lifestyle, filtration activity and easiness of sampling.

The aim of this study is to use mussels as bio-indicator of environmental contamination to investigate the presence of viruses in the Ravenna's harbor and in the stretch of the sea in front of it, considering spatial and temporal variability. Particularly, it was investigated the presence of human viruses like Norovirus (NoV) and Hepatitis A virus (HAV) to evaluate the anthropic impact on this area, and one of the most threatening finfish viral pathogen, the Nervous Necrosis Virus (NNV) responsible of high mortality outbreaks in farmed finfish during summer. The investigation showed the presence of human virus contamination only in mussels collected from the inner part of the harbor. Mussels showed a more frequent NoV contamination in cold months similarly to what observed in other areas. Genotyping showed the presence of multiple NoV strains representing those most frequently associated to foodborne human outbreaks and those most spread worldwide. Furthermore, it was detected the presence of HAV IA, a genotype frequently isolated from human infections associated with the consumption of raw seafood in Southern Italy. On the other hand, NNV contaminated mussels were detected in all investigated sites, including offshore gas platforms, showing a wide dissemination of this finfish pathogen. Most of the NNV contaminations has been detected during summer sampling. Genotyping showed high similarity of the detected viruses with NNV strains previously detected in finfish and bivalve mollusks of the Adriatic Sea. All together, these findings suggest that mussels could accumulate the NNV released by infected wild finfish host living this area.

1-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} (Bergh et al., 1989; Middleboe and Brussaard, 2017). They play an enormous role in ocean process through their interactions with all type of marine organisms. Accordingly, they can infect organisms ranging from the smallest type, like marine bacteria and archea to the largest marine mammals (Munn, 2006). Viruses form a central component of the "microbial loop" in ocean food webs (Munn, 2006) and several studies have shown that viruses are key players in the marine ecosystems; as they drive bacterial and algal mortality and evolution at nanoscale, they may influence the global-scale biogeochemical cycles and ocean productivity (Middleboe and Brussaard, 2017).

Viruses are also recognized as the causative agents of fish diseases (Middleboe and Brussaard, 2017), such as betanodaviruses that are responsible of the viral encephalo-retinopathy (VER) in several marine finfish species (Doan et al., 2017).

The VER, also known as viral nervous necrosis (VNN), is characterized by a vacuolating necrosis of neural cells of the brain, retina and spinal cord and causes up to 100% mortality in larval and juvenile finfish, and significant losses in older fish (Crane and Hyatt, 2011). The nervous necrosis viruses (NNV) have been reported in cultured marine finfish worldwide since the 1990s (Munn, 2006). They are small ssRNA virus of the genus *Betanodavirus*, family *Nodaviridae* (Thiery et al., 2012). Their genome consists of two molecules of RNA; the RNA1 that encodes a non-structural protein with RNA dependent RNA polymerase (RdRp) activity and the RNA2 that encodes the coat protein (CP; Crane and Hyatt, 2011; Doan et al., 2017). Based on a partial nucleotide sequence of the coat protein gene, betanodaviruses are divided into 4 species: *Striped jack nervous necrosis virus* (SJNNV), *Tiger puffer nervous necrosis virus* (TPNNV), *Barfin flounder nervous necrosis virus* (BFNNV) and *Redspotted grouper nervous necrosis virus* (RGNNV) (Thiery et al., 2012). Furthermore, reassortant strains have emerged from reassortment of RGNNV and SJNNV species and named 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; Olveira et al., 2009; Panzarin et al., 2012). VER is mainly observed in farmed finfish, although disease outbreaks have been also reported in wild finfish, mainly in groupers (Gomez et al., 2009; Vendramin et al., 2013). However, asymptomatic NNV infection has been detected in several wild finfish species, suggesting their possible role as carriers (Ciulli et al., 2007). Of further interest is the potential of wild finfish to become sub-clinical carriers as virus-contaminated water spreads from aquaculture enterprises into the marine environment (Gomez et al., 2010; Crane and Hyatt, 2011). Furthermore, the presence of

NNV was also detected in invertebrates in the Mediterranean Sea, South Korea and Japan (Gomez et al., 2008; Gomez et al., 2009; Ciulli et al., 2010; Panzarin et al., 2012). Particularly, NNV has been detected in clams, oyster and mussels (Volpe et al., 2018). The role of wild aquatic organisms such as bivalve mollusks in the interaction with finfish pathogens has been investigated (Volpe et al., 2017). Actually, no evidence for viral replication in invertebrates has been shown. Nevertheless, there is evidence indicating that, bivalve mollusks may act either as bio-filters or as reservoirs for finfish pathogens as a consequence of their ability to bio-accumulate microorganisms (Mortensen et al. 1992; Mortensen 1993; Paclibare et al. 1994; Skår and Mortensen 2007; Molloy et al. 2011; Pietrak et al. 2012; Wangen et al. 2012; Volpe et al., 2017). In particular, through experimental contamination trial, conducted on clams, it has been possible to point out that bivalve mollusks are able not only to accumulate NNV, but also to release alive viral particles. This confirms that bivalve mollusks could act as a source of viruses, posing a serious risk of outbreaks of VER in susceptible cultured finfish (Volpe et al., 2017).

The sea can also act as a reservoir for the transmission of viruses of human origin, especially the enteric viruses that contaminate coastal waters (Bosh et al., 2005; Munn, 2006). In fact, water-borne illnesses are the most often associated with viruses rather than bacteria (Brooks et al., 2005). Among these, the enteric viruses, originating from human excreta, cause a wide spectrum of illnesses in man, including hepatitis, gastroenteritis, meningitis, fever, rash, and conjunctivitis (Maalouf et al., 2010). Accordingly, the consumption of bivalve mollusks harvested from fecal polluted areas can pose a major risk factor for food-borne outbreaks (Henigman et al., 2015). Viruses, more frequently implicated in outbreaks are *Norovirus* and *Hepatitis A virus* (Maalouf et al., 2010).

Noroviruses (NoV) are non-enveloped, single-stranded positive RNA virus, member of the genus *Norovirus*, family *Caliciviridae* (Ilic et al., 2017). Viruses included in the genus *Norovirus* are highly diverse and 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 two genome fragments: the RNA-dependent RNA polymerase (RdRp) and the capsid protein (CP) genes (van Beek et al., 2018). Particularly, they have been described 14 GI and 29 GII RNA-dependent RNA polymerase (RdRp) genotypes and 8 GI and 23 GII capsid genotypes (Medici et al., 2014). In order to avoid confusion with capsid type nomenclature, RdRp genotypes are designated by a capital P (for "polymerase") followed by an Arabic number (Kroneman et al., 2013; Medici et al., 2014). Noroviruses are very stable in the environment (Ilic et al., 2017). They are the most common cause of human acute gastroenteritis (AGE) causing both sporadic and endemic illness across all age groups (Lindsay et al., 2015). Generally, noroviruses cause inflammation of the stomach or intestines and the main symptoms include vomiting or diarrhea

episode, with or without accompanying nausea, fever, or abdominal pain (Wang et al., 2018). Transmission can occur through contact with shedding person, food contamination during processing, preparation or serving, but also sewage-contaminated water used for consumption, cultivation or irrigation of food (Verhoef et al., 2010). In particular, the practice of consuming raw or undercooked bivalve mollusks, which come from contaminated shellfish-growing areas, has been frequently associated to NoVs infections (Maalouf et al., 2010; Henigman et al., 2015). Genotyping of norovirus detected in human can provide information about the origin of the outbreaks. Noroviruses GI are the most commonly associated with foodborne outbreaks (Verhloef et al., 2010). On the other hand, Noroviruses GII and particularly GII.4 are the most frequently associated to person-borne outbreaks (Verhloef et al., 2015). However, also some genotypes of norovirus GII have been associated to foodborne outbreaks (Vega et al., 2014). Furthermore, the presence of a single norovirus genotype is usually reported in clinical cases associated with direct contamination by food handlers, whereas, the presence of multiple genotypes may be detected when wastewaters are responsible for contamination of shellfish or other foodstuff (Kageyama et al., 2004; Le Guyader et al., 2008). Several investigations, in fact, pointed out the presence of more than one virus in bivalve mollusks (e.g. Norovirus GI and GII or Norovirus and HAV) (Kageyama et al., 2004; Croci et al., 2007). Norovirus outbreaks seem to have distinct seasonal activity (Lindsay et al., 2015). In particular, clear peak of norovirus outbreaks occurs during cold weather months on several continents, with lack of UV, cold temperature and frequent run-off (Maalouf et al., 2010; Campos and Lees, 2014).

Hepatitis A virus (HAV) is a hepatotropic agent from the genus *Hepatoviru*s, family *Picornaviridae* (Mbayed et al., 2002) and is responsible for acute viral hepatitis, which present a significant public health problem in many countries due to the persistent circulation of the virus in the environment and the possible contamination of water and food (Chironna et al., 2003). The Hepatitis A virus has been classified in seven different genotypes, which include human (I, II, III and VII) and simian (IV, V and VI) groups (Mbayed et al., 2002). Molecular epidemiology of HAV has been used to identify geographic or epidemiological sources of HAV isolates and the most of the human strains cluster in genotype I, which has been further divided into sub-genotypes A and B (Mbayed et al., 2002). The subgenotype IA remains the most widespread in the world (Robertson et al., 1992; Chironna et al., 2003). Enterically transmitted hepatitis viruses are distributed worldwide with no clear seasonal pattern, and transmission is linked to sanitary and living conditions of the population (Pintò and Saiz, 2007; Maalouf et al., 2010). Southern Italy, and in particular Puglia, is an endemic area for HAV infection contributing to the majority of Italian hepatitis A cases (Chironna M. et al., 2003) and this is related to the large consumption of raw mussels (Chironna et al., 2003).

The increased knowledge of marine viruses as pathogens and their importance in marine ecosystems may be attributed also to the application of powerful new tool and techniques (Munn, 2006). Studies show evidence of evolution of new strains, changes in virulence and transfer of viruses between ecosystems (marine to fresh water, fresh water to marine, and terrestrial to marine) (Munn, 2006). This shift, are often precipitated by movement of organisms between geographic areas which may occur naturally through migrations (Munn, 2006). However, also human activity has greatly enhanced global transport of marine species including pathogens (Harvell et al., 1999). In fact, habitat degradation and pollutant inputs, often brought about human activity, can facilitate disease outbreaks (Harvell et al., 1999). An example of pollutant inputs is the introduction into the marine environment of wastewaters only partially treated or not disinfected (Griffin et al., 2003), from different sources of discharged, disposal from boats, ballast water and contaminated river (Henigman et al., 2015). Although nowadays discharges comply with the current laws, their accumulation still raises concerns (Airoldi et al., 2016). The microbial quality of waters has been traditionally assessed by monitoring fecal indicator bacteria, such as *Escherichia coli*, intestinal enterococci and fecal coliform bacteria (Gonçalves et al., 2018; Liang et al., 2015). Nevertheless, several studies has been able to demonstrate that the bacterial indicator levels, used as standards were not predictive of viral pollution (Griffin et al., 2003; Brooks et al., 2005). In fact, one of the main limitations of coliform bacteria, as indicators of fecal pollution, is their poor correlation with the presence of non-bacterial pathogens, in particular with the presence of human viruses (Serratore et al., 2014; Gonçalves et al., 2018). Several studies had also pointed out that the enteric viruses are generally more resistant that bacteria to inactivation in water sources and are removed slowly from bivalve mollusks by depuration process (Croci et al., 2007; Ciulli et al., 2017) and this confirms that bacteria may not be found in contaminated water where viruses still persist (Brooks et al., 2005).

Mussels have been proposed as being useful for biomonitoring trace levels of contaminants in coastal water due to their wide distribution, sessile lifestyle, ease of sampling, resistance to stress and high accumulation of wide range of chemicals (Goldberg et al., 1978; Yap et al., 2012).

Bivalve mollusks are known as an important source of food for humans; they can filter several liters of seawaters daily and, due to this activity, they can accumulate both chemicals and microorganisms including pathogenic bacteria and viruses of public health concern (Croci et al., 2007; Serratore et al., 2014; Yap et al., 2012; Ilic et al., 2017). They have been identified as vectors of human enteric pathogen transmission more than 150 years ago (Maalouf et al., 2010). A previous study showed the presence of both HAV and NoV contamination in bivalve mollusks harvested in shellfish production areas of the northern Adriatic Sea (Croci et al., 2007).

The aim of this study is to use mussels as bio-indicator of environmental contamination to investigate, for the first time, the presence of viruses in the aquatic environment of Ravenna's harbor and in the stretch of the sea in front of it, considering spatial and temporal variability. Particularly were investigated: 1) the presence of human viruses like NoV and HAV in a such highly urbanized context subject to multiple anthropogenic uses; 2) the presence of one of the most threatening finfish pathogens like the NNV (*Betanodavirus*) in an area without a direct connection with susceptible finfish species farming areas. Results concerning the presence of investigated viruses and their characterization could contribute to increase the knowledge on their epidemiology and on the impact of this area of renowned environment, cultural and economic value.

2-Materials and method

2.1 Study area: Ravenna's harbor

Ravenna is the major coastal city of Emilia Romagna region 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 tourist seacoast (Airoldi et al., 2016). The seaside is protected by two converging dams, each one is 2400 meters long, while the side towards the city is close to the railway station (Lucialli et al., 2007).

The canal port is also directly connected with two lagoons which are comprised of the southern part of the Po Delta Park and included in the list of wetlands of international importance under the Ramsar Convention (Airoldi et al., 2016).

2.1.1 Harbor's history

The port of Ravenna was established thousand years ago, when Ravenna was closer to the Adriatic Sea than today. Due to its strategic geographic position and harbor characteristics it became an important Roman port. Even after the decline of the Western Roman Empire in the 5th century, the port kept active and entered in a golden age during the Byzantine domination (Airoldi et al.,2016).

The modern port of Ravenna was developed starting from 1737, when the previous one, southernmost, was abandoned due to excess of siltation (Gabbianelli et al., 2003).

The harbor evolution has also affected the surrounding coastal areas, as the construction of two large jetties (about 2400 m long each) to protected the harbor from siltation, altered the sediment transport and shaped the nearby tourist beaches (Airoldi et al., 2016).

After the II world war, Ravenna became an important international port when large gas fields were discovered offshore. Nowadays it is still one of the largest industrial and commercial seaports in Italy specialized in the handling of solid bulk, fertilizers, animal feed, general cargo, minerals and steel products (Airoldi et al., 2016).

2.1.2 Environmental setting

The area of the port of Ravenna develops in an alluvial sandy system within the Po River Valley, strongly influenced by recent changes in the sea level, by the sediment load of many rivers and many human hydraulic interventions. It is an area of renowned environment, cultural and economic value and one of the world's leading holiday destinations (Airoldi et al., 2016).

The Emilia Romagna's coastal areas are naturally sedimentary. The seabed is gently sloping (about $6m \ km^{-1}$) and a wide surf zone is exposed to waves raised by prevailing south-easterly and north-easterly winds (Bondesan et al., 1995; Colosio et al., 2007). Shallow subtidal sediments comprised well sorted fine to medium sand and are colonized by macrofaunal assemblages generally dominated by bivalve mollusks (Bertasi et al., 2007).

Offshore gas platforms and terminals also 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 maximum depth of the 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).

There are two main lagoons, south and north of the canal port, named Pialassa Piomboni (3km) and Pialassa Baiona (10 km), respectively (Airoldi et al.,2016). They are connected with the port by channels and they are both included in the National Park of Po river and in the list of wetlands of international importance under the Ramsar Convention (Airoldi et al., 2016). In recent years Pialassa Baiona has also been recognized as a "sensitive area" and has been included in the European Special Protection Areas (SPAs, Bird Directive 79/409/EEC) and in the proposed sites of Community interest (pSICs, Habitat directive 92/43/EEC). Contrariwise, the Pialassa Piomboni is now a section of Ravenna's harbor without any naturalistic interest (Airoldi et al., 2016).

Today the Pialassa Baiona has a total extension of about 1.100 ha (Vistoli, 2003) and is formed by semi-submerged areas and shallow waters traditionally known as "clear" bordered by artificial banks (Gabbianelli et al., 2003). The clear path are delimited and connected each other by a network of canals and drains (Gabbianelli et al., 2003), and many of which are navigable by small boats (Airoldi et al., 2016). The main channels have depths of 2-3 meters even if in some places they can reach 5

meters. The minor channels instead have a minimum depth of about 1 meter (Farina et al., 1994). Along the canals and drains there have been installed numerous sluice gates, overflow devices (weirs), which allow a certain artificial regulation both inlet flows and, in part, in the internal circulation (Farina et al., 1994). Lagoons are connected to the sea by a single outlet represented by the Candiano canal and by the outer harbor (Gabbianelli et al, 2003; Fig. 1). Complete water turnover in the lagoon has been estimated to take on average 3 days (Ponti et al., 2005; Airoldi et al., 2016) and it depends on the inputs and outputs of the tidal flow and by fresh water inputs (Gabbianelli et al., 2003). The lagoon receives water inputs from five main channels that come from both urban and agricultural areas (Airoldi et al., 2016). Bottoms are prevailingly sedimentary, ranging from mud to fine sand (Airoldi et al., 2016) and species diversity is relatively high, even if hypoxic events occurring in summer period lead to a transitory reduction in the number of species (Ponti and Abbiati, 2004). The Pialassa Baiona lagoon also harbors several recreational and educational activities including fishing, hunting, hiking, birdwatching, canoeing and school educational excursions (Vistoli, 2003).
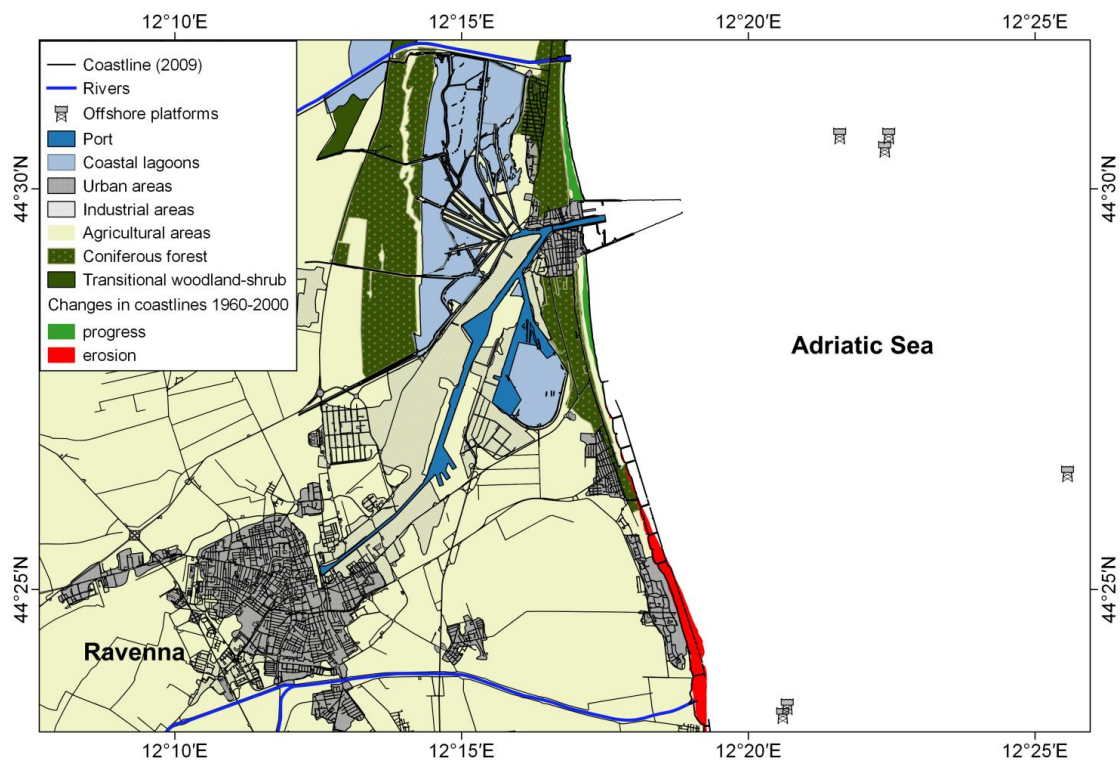

Fig. 1 Port of Ravenna and surrounding areas. Land cover modified from CORINE, 2006; coastal erosion data from the national geoportal of the Italian Environmental Ministry; road and hydrographic networks from Emilia-Romagna Region geoportal; offshore platforms from the nautical map of the Hydrographic Institute (Mercator projection, datum WGS84).

2.1.3 Economic setting

The port of Ravenna is one of the most important ports in Italy as far as solid bulk is concerned, including raw materials for the ceramics, grains, fertilizers, animal feed and flour industries, minerals, iron and steel products, coal and timber, containers and general cargo. It is also important for liquid bulk cargoes, including refined products, gas and crude oil (Airoldi et al., 2016).

In 2017, the port of Ravenna handled over 26.5milion tons of cargo, including 22.6 million tons of imports and 3.8 million tons of exports. Solid bulk cargoes of 11.4 million tons; Liquid bulk cargoes of 4.5 million tons and general cargoes of 10.5 million tons (Ravenna Port Authority[1]). Operations and activities are controlled, planned, promoted by the Port Authority of Ravenna even if many facilities and services are provided by private interests. The terminals have direct rail and road connections with regular service to Milano, Modena and Bologna (World port source[2]).

The ferry terminal occupies an area of 125 m^2 and offers two berths for ferries with canal bed depth of 11.5 m. it can accommodate ships of 261 m length and 9.6 m draft (Airoldi et al., 2016). At the moment, the ferries operating in the terminal are serving the Ravenna-Catania and the Ravenna-Catania-Brindisi routes operated by Tirrenia and Grimaldi respectively. In 2011 a new cruise passenger terminal was established at Porto Corsini; it is able to serve cruise ship up to 350 m long with 10.5 m draft (Airoldi et al., 2016).

The port of Ravenna also harbors some marinas, including the largest one of the Adriatic Sea, MARINARA with over 1150 boat docks and neighborhood support intense boating and sporting activities (Airoldi et al., 2016).

Following coastal development and environmental degradation, fishery became a marginal economic activity in the region and nowadays the role of fishery in local economy is negligible. Main fishing activity in the open sea include the harvesting of marine clams (*Chamelea gallina*) by using fishing dredges, the harvesting of mussels (*Mytilus galloprovincialis*) from offshore platforms by surface supplied divers and fishery by trawling (Pranovi, 2000; Airoldi et al., 2016). The small local fishery in the lagoons includes the harvesting of the invasive Manila clams (*Ruditapes philippinarum*) and cuttlefish (*Sepia officinalis*; Airoldi et al., 2016). Moreover, some fish farms are present in other lagoons along the Adriatic coast; however, no direct connection exists between these lagoons and the Ravenna's harbor. The main reared finfish species are European seabass (*Dicentrarchus labrax*), the gilthead seabream (*Sparus aurata*) and the European eel (*Anguilla Anguilla*; Airoldi et al., 2016).

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[1] http://www.port.ravenna.it/anno-2017
[2] http://www.worldportsource.com/ports/commerce/ITA_Port_of_Ravenna_1171.php

2.2 Sampling design and field activities

First, an explorative sampling was conducted in March. The area of the Ravenna's harbor and the connected lagoons was checked for the presence of bivalve mollusk species suitable for sampling and virus detection. Mussel has been identified as the most abundant bivalve mollusk species suitable for virus detection. Accordingly, mussel samples (*Mytilus galloprovincialis* and *Xenostrobus securis*) have been collected from three sites along the Ravenna's harbor and subjected to a preliminarily virus detection (HAV, NoV, NNV).

Given the high presence of viruses in mussels collected during the explorative sampling a more structured sampling was carried out.

The sampling had the aim to investigate, for the first time, the presence of viral contamination in mussels of Ravenna's harbor, considering different sites of collection and different time points.

To investigate the whole harbor canal, four sampling sites have been established, two located in the outer part of the port (ST.1, ST.2), and more exposed to the marine weather conditions, and two located in the innermost area (ST.5, ST.6), near the center of Ravenna (Fig. 2). The sampling was conducted every two months from May 2018 to January 2019. During the summer period (July and September) the sampling was extended to the stretch of the sea in front of the harbor collecting samples from offshore gas platforms (Fig. 3). During this period, in fact, platforms are subjected to periodical cleaning by removing mussels from support pylons, these mussels are sold for the human consumption. At each time (July and September), mussels were collected from two different platforms (Fig. 3). After collection, mussels sampled were maintained under refrigerated conditions and immediately transferred to the laboratory to be processed for viral detection.

2.2.1 Physical-chemical parameters

During each sampling, physical-chemical water parameters (temperature, salinity, pH, Redox, dissolved oxigen) have been collected. Temperature and salinity has been measured at the time of the sampling using a conductivity meter-thermometer (HD9213, Delta OHM). Where practicable (ST.1 and ST.6) mean daily values have also been collected using temperature probes set to store a data every 10 min (Star-Oddi, DST Centi-T). This measurement was done to get a more accurate and less conditioned, by the time of the sampling, temperature values. The mean daily temperature values have been collected also to compare the difference between the two further apart sites (ST.1 and ST.6). The pH-redox potential have been measured using a pH meter (HI98121, Hanna Instrument). A paired-samples 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 for accepted statistical significance was $p < 0.05$.
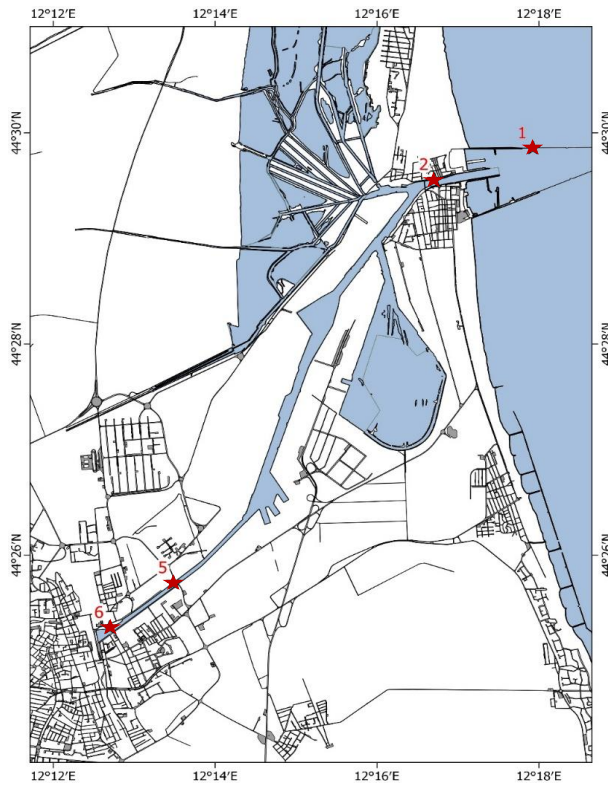
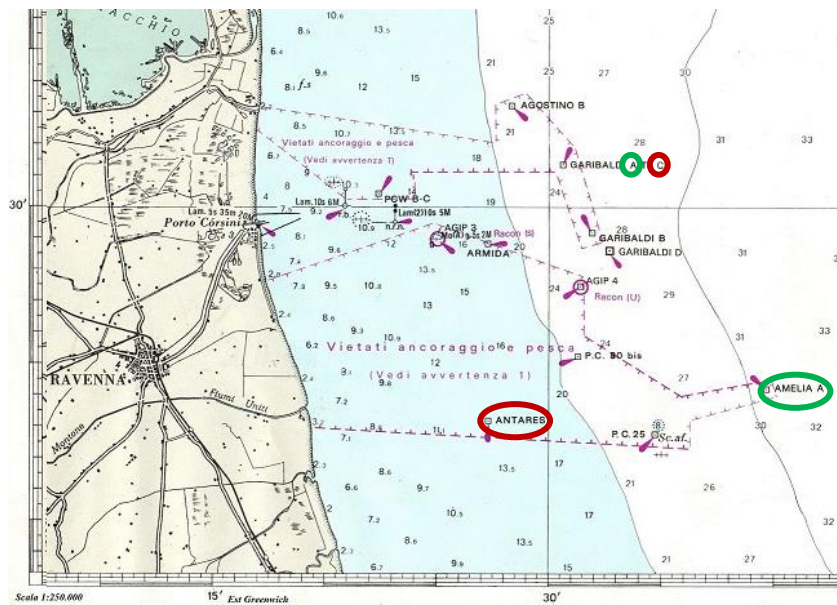Fig. 2 Sampling Area (Mercator projection, datum WGS84).

Fig. 3 Gas platforms sampled. Red circles show platform sampled in July and green circles show platform sampled in September (Mercator projection, datum WGS84; Mancini, 2012).

2.3 Viral detection

2.3.1 RNA extraction

The mussel hepatopancreas samples were collected from specimens, homogenized and treated with proteinase K (Sigma, St. Louis, MO, USA) and then the RNA was extracted from an aliquot of 100 µl of supernatant, using the commercial kit NuceloSpin RNA (Macherey-Nagel, Düren, Germany). To lysate cells 400 µl of Buffer RA1 and 4 µl of 2-mercaptoethanol were added to each sample, and mixed by vortexing. To reduce viscosity and clear the lysate a first filtration of samples was performed using the NucleoSpin Filter and centrifuged for 1 min at 11,000xg at room temperature. After discarding the NucleoSpin filter, 350 µl of ethanol (70%) were added to the lysate and mixed by pipetting up and down (5 times). To bind RNA, for each sample was used a NucleoSpin RNA Column. In particular, the lysate was loaded to the column and centrifuged for 1 min at 8,000xg at room temperature. Then the column was placed into a new collection tube. To dry the membrane 350 µl of MDB were added and the column was centrifuged for 1 min at 11,000xg. To remove DNA it was prepared a DNase reaction mixture adding 10 µl of reconstituted rDNase to 90 µl of Reaction Buffer for rDNase; 95 µl of this solution were applied directly onto the center of the silica membrane of the column, and then incubate at room temperature for 15 min. To wash and dry the silica membrane, 200 µl of Buffer RAW2 were added to the NucleoSpin RNA Column and centrifuged for 1 min at 11,000xg. After placing the column into a new collection tube, 600 µl of Buffer RA3 were added to the column and centrifuged for 1 min at 11,000xg. The collection tube was discarded and then the column was placed back in the same tube, added 250 µl of RA3 and centrifuged for 3 min at 11,000xg. For the RNA elution, the column was placed in a new nuclease-free collection tube and 60 µl of Nuclease-free water was added to the column and centrifuged for 1 min at 11,000xg. RNA samples were stored at -80°C until use.

2.3.2 Hepatitis A virus detection

The presence of HAV viruses was investigated by a RT-nested PCR method using specific primers (Le Guyader et al., 1994). Briefly, the first amplification step was conducted through a one-step RT-PCR assay with primers AV1 (GGAAATGTCTCAGGTACTTTCTTTG) and AV2 (GTTTTGCTCCTCTTTATCATGCTATG), using the SuperScript III One-step RT-PCR System (Invitrogen, Carlsbad, USA). The reaction mixture contained 1x Reaction Mix, 0.8 µM for each primer, 0.3 µl Superscript III/Platinum Taq enzyme mix and 3 µl RNA in 15 µl total volume.

The thermal cycling conditions were 45°C for 30 min, 95 °C for 2 min, followed by 40 amplification cycles of 94 °C for 35s, 55°C for 1 min s and 72 °C for 75s. A final extension was performed at 72°C for 7 min. Semi-nested PCR was conducted with primers AV2 and AV3

(TCCTCAATTGTTGTGATAGC), using the Platinum Taq DNA polymerase (Invitrogen). The reaction mixture contained 1x PCR buffer, 1.5 mM MgCl2, 200mM dNTPs, 0.5 µM for each primer, 1 unit of Platinum Taq DNA polymerase, nuclease-free water and 1 µl of RT-PCR diluted in 25 µl total volume. The thermal cycling conditions were 95 °C for 5 min, followed by 40 amplification cycles of 94 °C for 45s, 55°C for 45s and 72 °C for 60s. A final extension was performed at 72 °C for 7 min.

The PCR product obtained from these reactions were visualized through gel electrophoresis. The gel was made by 1.5% agarose. In each run, a reference molecular marker (100 bp DNA molecular marker, Invitrogen, Carlsbad, USA) has been uploaded for the identification of the PCR product size.

2.3.3 Norovirus detection

The presence of Noroviruses was investigated through a RT-qPCR method with specific primers and probes. Primer QNIF4 (5'-CGTGGATGCGNTTCCAT-3'; Da Silva et al., 2007), Primer NV1LCR (5'-CCTTAGACGCCATCATCATTTAC-3'; Svraka et al., 2007), Probe NVGG1p (5'-FAM-TGGACAGGAGAYCGCRATCT-3'TAMRA; Svraka et al., 2007); Primer QNIF2 (5'-ATGTTCAGRTGGATGAGRTTCTCWGA-3'; Loisy et al., 2005), Primer COG2R (5'-TCGACGCCATCTTCATTCACA-3'; Kageyama et al., 2003), Probe QNIFS (5'-FAM-AGCACGTGGGAGGGCGATCG-3'TAMRA; Loisy et al., 2005) targeting GI and GII respectively. The reaction mixture contained 1x Buffer Mix, 0.5 µM for primer forward, 0.9 µM for primer reverse, 0.25 µM for the probe, 500 nM Rox, nuclease-free water, 0.25 µl ThermoScript/Platinum Taq enzyme mix and 2.5 µl RNA in 12.5 µl total volume. The thermal cycling conditions were 55°C for 30 min, 95°C for 1 min, followed by 45 amplification cycles of 95°C for 15s, 60°C for 60s and 65°C for 60s. Samples that resulted positive to RT-qPCR were further processed to genetically characterize the detected norovirus. A viral fragment was amplified by a RT-nested PCR method,that amplify both genogroups GI and GII, and then sequenced. Briefly, the first amplification step was conducted through a one-step RT-PCR assay with primers JV12 (ATACCACTATGATGCAGATTA; Vinjé and Koopmans, 1996) and JV13 (TCATCATCACCATAGAAAGAG; Vinjé and Koopmans, 1996) using a SupersScript III One-Step RTPCR with Platinum Taq (Invitrogen). The reaction mixture contained 1x Reaction Mix, 0.8 µM for each primer, 0.3 µl Superscript III/Platinum Taq enzyme mix and 3 µl RNA in 15 µl total volume. The thermal cycling conditions were 48°C for 30 min, 95°C for 5 min, followed by 40 amplification cycles of 94°C for 30s, 46°C for 30s and 72°C for 60s. A final extension was performed at 72°C for 10 min. To avoid any cross-contamination the amplification reactions were all set up with negative controls. Semi-nested PCR was conducted using primers NVG1 (TCNGAAATGGATGTTGG; Green et al., 1998) or NVG2 (AGCCAGTGGGCGATGGAATTC;

Boxman et al., 2006) targeting GI and GII respectively, using the Platinum Taq DNA polymerase (Invitrogen). The reaction mixture contained 1x PCR buffer, 1.5 mM MgCl2, 200 µM dNTPs, 0.44 µM of each primer, 1 units of Platinum Taq DNA polymerase, nuclease-free water and 1 µl of the product of the RT-PCR in a 25 µl total volume. The thermal cycle consisted of 95°C for 5 min and of 35 amplification cycles of 94°C for 30s, 46°C for 30s and 72°C for 60s. A final extension was performed at 72°C for 10 min. To avoid any cross-contamination, negative controls were run along with all reactions. The PCR product obtained from these reactions were visualized through gel electrophoresis. The gel was made by 1.5% agarose. In each run a reference molecular marker (100 bp DNA molecular marker, Invitrogen, Carlsbad, USA) has been uploaded for the identification of the PCR product size, obtained with the reaction.

2.3.4 Nervous Necrosis Virus detection

The presence of Necrosis Nervous Virus was investigated by two RT-nested PCR method, using primers targeting the viral RNA1 (Toffolo et al., 2007) and RNA2 (Ciulli et al., 2006) segments.

Briefly, the first amplification step was conducted through one-step RT-PCR assay with primers VNNV5 (5'-GTTGAGGATTATCGCCAACG-3') and VNNV6 (5'-ACCGGCGAACAGTATCTGAC-3'; Toffolo et al., 2007) or S6 (5'-ATGGTACGCAAAGGTGATAAGAAA-3') and S7 (5'-GTTTTCCGAGTCAACACGGGT-3'; Ciulli et al., 2006) targeting the RNA1 and RNA2 viral genome respectively, using the SuperScript III One-Step RT-PCR System (Invitrogen, Carlsbad, USA).

The reaction mixture contained 1x Reaction Mix, 0.8 µM for each primer, 0.3 µl Superscript III/Platinum Taq enzyme mix and 3 µl RNA in 15 µl total volume. The thermal cycling conditions were 45°C for 30 min, 95°C for 2 min, followed by 40 amplification cycles of 94°C for 60s, 58°C for 60s and 72°C for 60s. A final extension was performed at 72°C for 7 min. To avoid any cross-contamination the amplification reactions were all set up with negative controls. Semi-nested PCR was performed for RNA1 amplification with primers VNNV6 and VNNV7 (5'-CACTACCGTGTTGCTG-3'; Toffolo et al., 2007) while nested PCR for RNA2 was conducted with primers F2 (5'-CGTGTCAGTCATGTGTCGCT-3') and R3 (5'-CGAGTCAACACGGGTGAAGA-3'; Nishizawa et al., 1994) using the Platinum Taq DNA polymerase (Invitrogen). The reaction mixture contained 1x PCR buffer, 1.5 mM MgCl2, 0.25 µM of each primer, 1.25 units of Platinum Taq DNA polymerase, nuclease-free water and 1 µl of the product of the RT-PCR 1:100-diluted in a 25 µl total volume. The thermal cycle consisted of 95°C for 5 min and of 40 amplification cycles of 94°C for 30s, 57°C or 56°C (RNA1 and RNA2 respectively) for 30s and 72°C for 30s. A final extension was performed at 72°C for 7 min. To avoid any cross-contamination, negative controls

were run along with all reactions. The PCR products obtained from these reactions were visualized through gel electrophoresis. The gel was made by 1.5% agarose. In each run, a reference molecular marker (100 bp DNA molecular marker, Invitrogen, Carlsbad, USA) has been uploaded for the identification of the PCR product size, obtained with the reaction.

2.3.5 Genotyping

Samples that were positive to the viruses in RT-PCR or nested/eminested PCR were purified, quantified and sequenced. The purification of the PCR products has been conducted using the Exosap reagent (Affymetrix, Santa Clara, USA) that is able to remove dNTP and primers residue of the amplification reaction. The PCR purified product have been quantified using the fluorometer Qu-bit (Invitrogen, Carlsbad, USA) comparing the fluorescence to the reference standards. The sequences of the purified PCR products were obtained through the sequencing service Bio-Fab Sequencing Service (Rome, Italy). For each sample has been prepared a mixture containing a pre-established quantity of DNA and a specific primer. The sequences obtained in the form of ferograms have been corrected manually and analyzed through the online program Basic Local Alignment Search Tool (BLAST), available on the National Center for Biotechnology Information site[3], 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 a selection of Italian HAV strains (Chironna et al., 2003) available in GenBank[4] using Clustal W implemented in the BioEdit software[5]. Neighbor-joining phylogenetic analysis of the partial VP1 gene was performed with MEGA 7 software[6]. Bootstrap analysis was carried out on 1,000 replicates. The genotyping of noroviruses detected in mussels have been conducted using the Norovirus Typing Tool Version 2.0 (Kroneman et al., 2011).

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 vertebrate and invertebrates as well as with betanodavirus reference strains available in GenBank[4] using Clustal W implemented in the BioEdit software[5]. Maximum-likelihood phylogenetic analysis of the partial RNA 1 and RNA 2 was performed with MEGA 7 software[6]. Bootstrap analysis was carried out on 1,000 replicates.

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[3] http://blast.ncbi.nlm.nih.gov/Blast.cgi
[4] www.ncbi.nlm.nih.gov
[5] http://bioedit.software.informer.com/
[6] www.megasoftware.net

3-Results

3.1 Physical-chemical parameters

The salinity showed values ranging between 18.6 psu and 27.4 psu. The pH presented values ranging between 7.80 and 8.78. The Redox potential, showed values ranging between 18 mv and 145 mv and the dissolved oxygen presented values ranging between 4.9 mg/L and 8.9 mg/L. All data collected are reported in the supplementary materials (Table S1).

The temperature measured during each time of sampling showed values ranging between 7 °C and 28.1 °C (Fig. 4). The mean daily temperatures collected at ST.1 and ST.6 showed values ranging between 7°C and 31 °C (Fig. 5). The temperature trends detected with the two methods were entirely overlapping (Fig. 4 and 5). Regardless of site position, the temperature values of all the sampling sites showed a high variability depending on the seasons with the highest temperature in July and the lowest temperature in January (Fig. 4). Unfortunately, the probe at ST.1 was lost due to an exceptional storm occurred in November; however, it has been possible to compare temperature values of these two sites for a four months period (May-September). The comparison between the values measured in the two sites showed a ΔT between -1.68 and 2.73 °C; the analysis of data showed that the inner site (ST.6) was on average 1.03 °C ± SD 0.94 ($p < 0.01$) warmer than the external one (ST.1; Fig. 6). The pairwise comparison between temperatures collected at different sites at each sampling time confirmed the previous analysis.
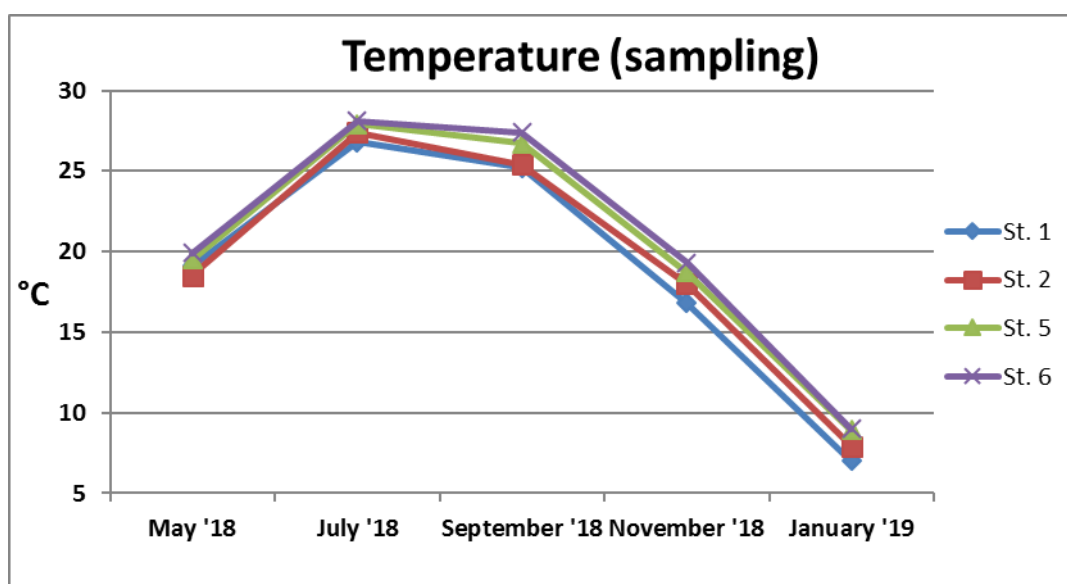

Fig. 4 Temperature values collected at each time of sampling.
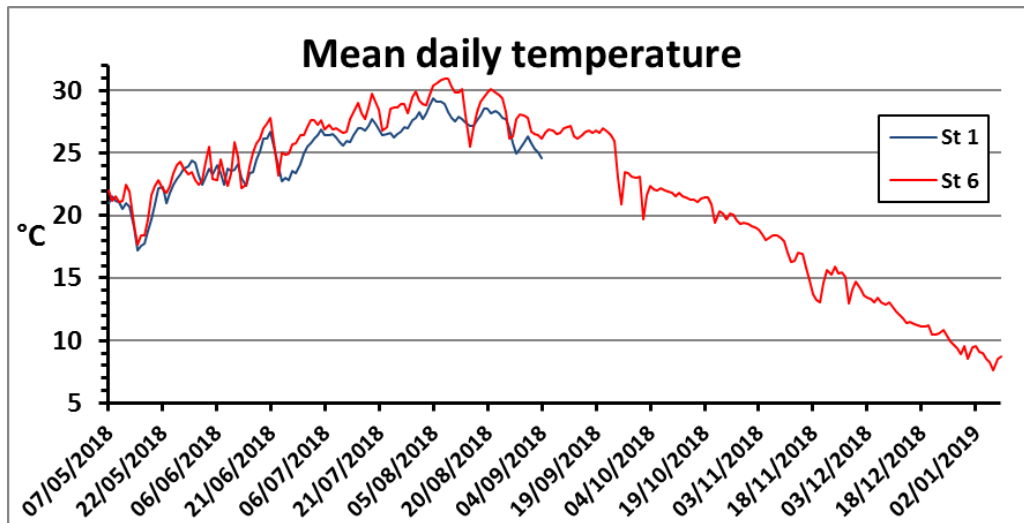
Fig. 5 Mean daily temperature values of ST.1 and ST.6 (May-January).
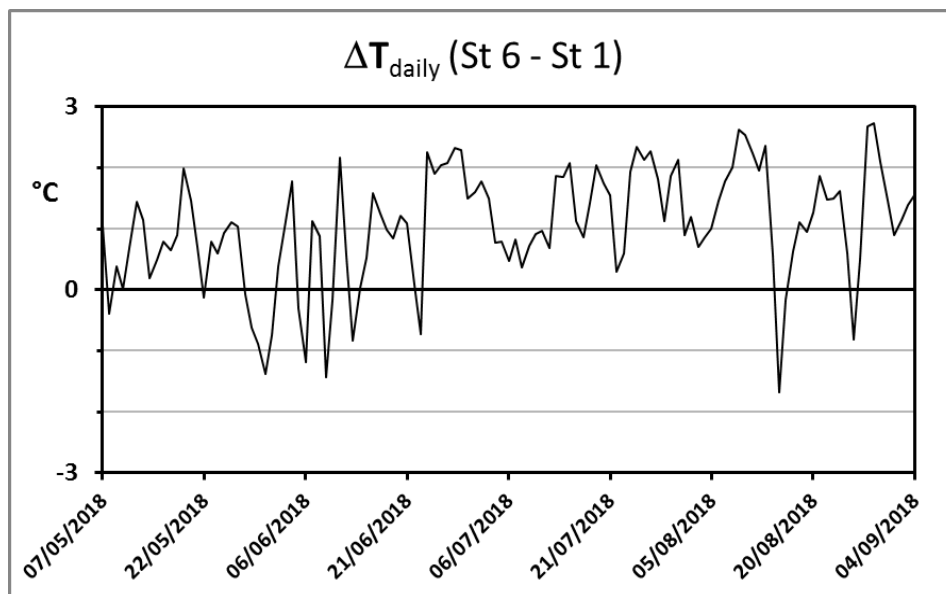
Fig. 6 ΔT values of ST.1 and ST.6 (May-September).

3.2 Explorative survey

All investigated viruses have been detected in Ravenna's harbor during the explorative survey. Particularly, HAV has been detected in two out of the three investigated sites, NoV has been detected in all three sites and NNV has been detected in two sites out of three. Genotyping of viruses detected in these positive samples are reported in the genotyping sections.

3.3 Survey May 2018-January 2019

The investigations carried out in the Ravenna's harbor showed the presence in mussels of both human and finfish pathogenic viruses.

3.3.1 Human viruses

Twenty five percent of mussel samples collected from Ravenna's harbor resulted contaminated by HAV or NoV. Particularly, 50% of samples collected from the inner sites showed to be contaminated, whereas none of those collected from the outer sites showed viral contamination. Moreover, no viral contamination were detected in mussel samples collected from the gas platforms in July and September. However, considering number of positivity per sampling, none of the mussel samples collected in July and September resulted contaminated. On the contrary, 50% of samples were contaminated in May and January and 25% in November.

Detailed results concerning human virus contamination detected in mussels are reported in table 1. Both human viruses investigated in this study has been detected in mussels collected in the innermost area of the port, near the city center of Ravenna (ST.5, ST.6). In particular, the hepatitis A virus (HAV) was found in one site (ST.6) in May. Norovirus has been found in both inner sites (ST.5, ST.6), in May, November and January.

Table 1. Presence of human viruses in the sampled sites.

	May	**July**	**September**	**November**	**January**
ST.1	Negative	Negative	Negative	Negative	Negative
ST.2	Negative	Negative	Negative	Negative	Negative
ST.5	**NoV**	Negative	Negative	Negative	**NoV**
ST.6	**NoV, HAV**	Negative	Negative	**NoV**	**NoV**

3.3.1.1 Genotyping HAV

A VP1 gene fragment has been sequenced for HAV samples detected during explorative survey (March, two positives) and May sampling (one positive). The three sequences showed a 100% nucleotide identity. The detected HAV strain showed the highest nucleotide identity (98-99%) with sequences of HAV subtype IA (Genbank accession numbers: MF416223; AY441441; AJ505803; AJ505800); some of these strains have been already isolated from water and also from human with infection associated to the consumption of raw seafood (Chironna et al., 2003).

Phylogenetic analysis of HAV detected strains confirmed that they belong to the subtype IA (Fig. 7).
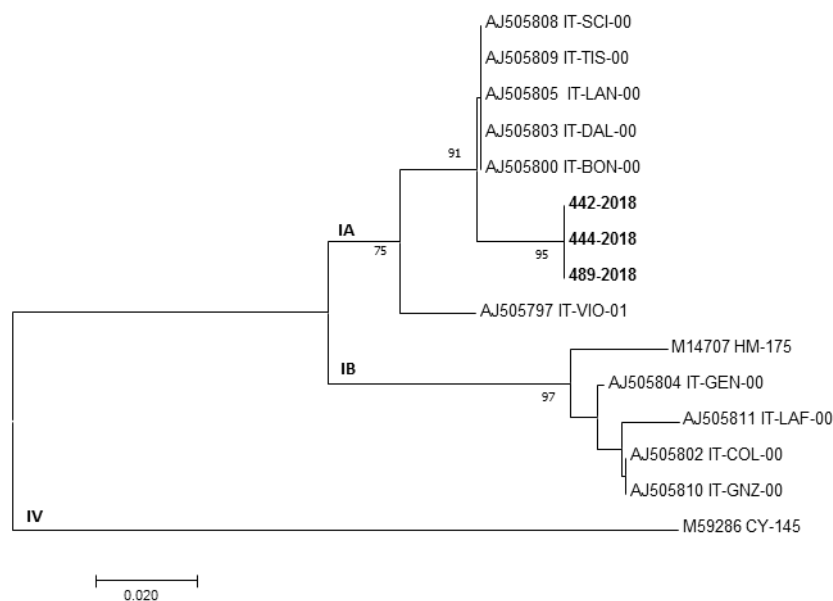

Fig. 7 Neighbor-joining phylogenetic tree based on the partial VP1 nucleotide sequences (185 bp). Sequences retrieved from GenBank are reported with the isolate name and accession number. Bootstrap values >70% are shown. Branch lengths are scaled according to the number of nucleotide substitutions per site. The scale bar is reported.

3.2.1.2 Genotyping norovirus

The use of a genogroup-specific RT-qPCR showed the presence of both norovirus genogroups. Considering all tested samples positive to NoV (n=8; including both explorative and structured surveys), 87.5% (n=7) of positive mussels had NoV GI contamination and 100% had NoV GI contamination. Most of the samples (n=7), in fact, showed to be co-contaminated by both GI and GII noroviruses.

For seven samples, an RdRp fragment was sequenced and characterized to genotype level. Concerning genogroup I, it was detected the presence of the genotypes GI.P2 (n=2) and GI.P4 (n=1),

whereas regarding genogroup II, it was detected genotypes GII.P4 (n=1), GII.P17 (n=1), GII.P21 (n=1) and GII.Pe (n=1). The genotyping showed a high variability of NoV variants present in the investigated area.

3.3.2 Nervous Necrosis Virus

Samples that showed positivity to at least one of the two PCR reactions (RNA 1 or RNA2) were considered positive to NNV.

Thirty five percent of mussel samples collected from Ravenna's harbor resulted contaminated by NNV. The presence of NNV has been found in all the sites sampled. Particularly, 30% of samples collected from the inner sites (ST.5 and ST.6) and 40% of those collected from the outer sites (ST.1 and ST.2) showed to be contaminated by NNV. Moreover, 50% of samples collected from gas platform were contaminated. Considering the number of positivity per sampling, none of the mussel samples collected in November and January resulted contaminated. On the contrary, 50% of samples collected in May and July and 66.6% in September were contaminated.

Detailed results concerning NNV contamination detected in mussels are reported in table 2.

Table 2. Presence of NNV in sampled sites

NODAVIRUS	May	July	September	November	January
ST.1	Negative	**Positive**	**Positive**	Negative	Negative
ST.2	Negative	**Positive**	**Positive**	Negative	Negative
ST.5	**Positive**	Negative	Negative	Negative	Negative
ST.6	**Positive**	**Positive**	Negative	Negative	Negative
Platform GAR A/C	n.d.	Negative	**Positive**	n.d.	n.d.
Platform ANATARES/AMELIA	n.d.	Negative	**Positive**	n.d.	n.d.

n.d. not determined

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

The phylogenetic analysis of the RNA 1 showed that NNV detected in mussels clustered in various subgroups of RGNNV genotype. Furthermore, for two samples collected in July and September, it was possible to detect a co-contamination from two different NNV viral strains (Fig. 8).

Fig. 8 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.

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The phylogenetic analysis of RNA2 showed that most of the NNV from bivalve mollusks clustered with RGNNV genotype, however, two viruses detected in mussels collected in March and May clustered with the SJNNV genotype (Fig. 9), showing to be RGNNV/SJNNV reassortants.
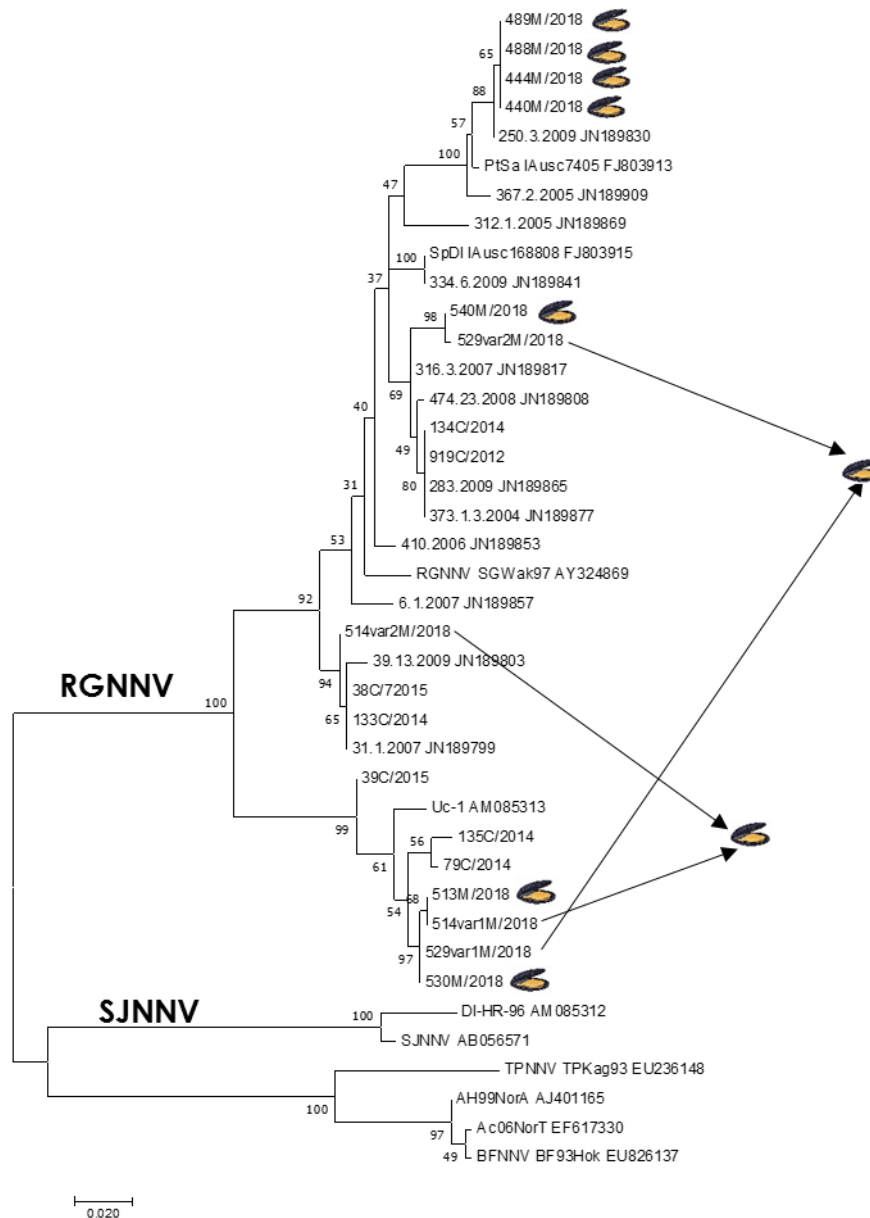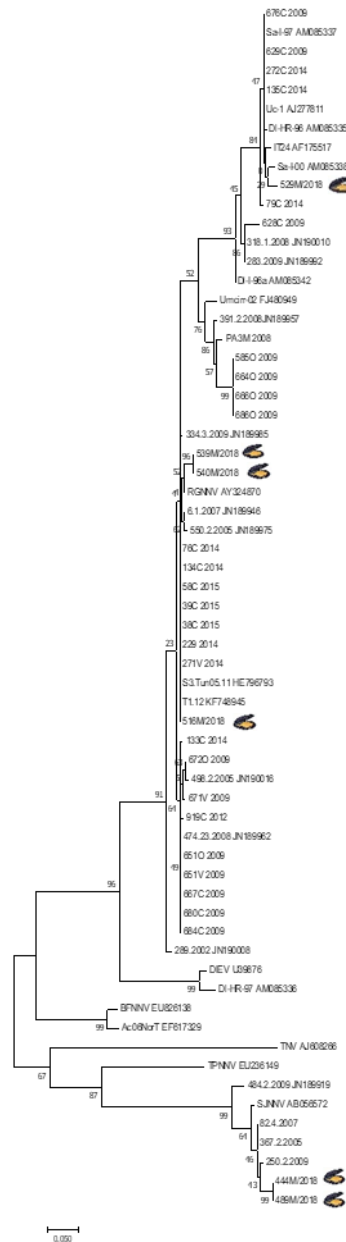

Fig. 9 Maximum likelihood phylogenetic tree based on partial RNA2 nucleotide sequences (281 bp). Sequences retrieved from GenBank are reported with the isolate name and accession number. Bootstrap values >70% are shown. Branch lengths are scaled according to the number of nucleotide substitutions per site. The scale bar is reported.

4-Discussion and conclusions

The Ravenna's harbor it is an area of cultural and economic value (Airoldi et al., 2016) but human pressure on the coast has been historically intense leading to severe urbanization and overexploitation of natural resources and nowadays most wetlands and lagoons have been filled by sediments or reclaimed (Cencini, 1998; Airoldi and Beck, 2007; Lotze et al., 2011). Accordingly, this area suffers several problems that are typical of the urbanized environments, including loss of habitat, loss of species, introduction of alien 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 water from several industrial plants. Although nowadays discharges comply with the current laws, their accumulation still raises concerns (Airoldi et al., 2016). Enteric viruses originating from human excreta may enter into the environment through the discharge of waste materials from infected individuals. The enteric viruses may cause a wide spectrum of illnesses in humans, including gastroenteritis, hepatitis, fever, conjunctivitis. Particularly, the viruses most frequently associated to outbreaks are hepatitis A virus and norovirus (Maalouf et al., 2010).

The viral investigation conducted in this study showed the presence of several viruses in the Ravenna's harbor and this, confirms also the usefulness of using mussels as bioindicators of viral environmental contaminants.

Particularly, 25% of the analyzed samples were contaminated by human pathogens (HAV and NoV). A similar prevalence (22%) has been previously found in shellfish harvesting areas in the North Adriatic Sea (Croci et al., 2007). However, in our study, the presence of both human viruses HAV and NoV has been found only in the inner sites of the harbor, while no human viruses have been found in the outer sites and in the mussels collected from offshore gas platforms. These findings suggest that the detection of human viruses in the area closest to the city center could be attributed to the presence of drains and wastewaters that may introduce these pathogens in the port environment. Several studies have already shown that the discharge into the marine environment of wastewaters can facilitate the introduction of human enteric viruses (Maalouf et al., 2010; Henigman et al., 2015; Gonçalves et al., 2018).

Regarding HAV, the strains detected in this study belong 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 (Costantino et al., 2017). Genotyping and molecular epidemiology of HAV have been used to identify geographic or epidemiological sources of HAV isolates (Mbayed et al., 2002). Particularly, 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 (foodborne vs person-borne) and to differentiate foodborne outbreaks caused by food contamination early in the food chain from those caused by food handlers contamination food (Verhoef et al., 2010). Particularly, 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 more often associated to person-borne outbreaks (Verhoef et al., 2015).

In this study, both genogroups (GI and GII) have been found. Furthermore, the genotyping characterization allowed to show the presence of genotypes GI.P2 and GI.P4. Norovirus GI.P2 has already been found in mussels collected from harvesting areas in Slovenia and associated with the consumption of raw seafood in China (Wang et al., 2015; Henigman et al., 2015). Regarding GII, genotyping has shown the presence of GII.P4, GII.P17, GII.P21 and GII.Pe. A previous study pointed out the presence of several norovirus GII.P4 and GII.P21 in mussels collected in Slovenia (Henigman et al., 2015). Moreover, surveillance data from the NoroNet network showed that GII.P4 and GII.Pe represents the genotypes most frequently detected worldwide inside the GII genogroup. Particularly, GII.P4 was the dominant genotype since 2005 until 2013 when GII.Pe became more prevalent (van Beek et al., 2018). On the contrary, GII.P17 emerged only recently (in Asia in 2014), but spread very quickly all over the world and circulated widely in Europe in 2015-2016 (van Beek et al., 2018). At present, GII.Pe, GII.P4 and GII.P17 represent the most frequent norovirus genotypes detected worldwide and in this respect, our virological investigation using mussels of Ravenna's harbor reflects this scenario.

The virological investigation conducted showed also an HAV and NoV co-contamination and frequent NoV GI-GII co-contaminations in the same sample. The presence of co-contaminations have been shown in bivalve mollusks in several studies (Kageyama et al., 2004; Croci et al., 2007). Particularly, viral mixed shellfish contaminations have already been associated with the presence of both human and environmental pollution in the mussel farming areas (Ilic et al., 2017).

Another important issue regarding human virus contamination in bivalve mollusks is the seasonality occurrence and this is due to several factors. Previous studies have pointed out that norovirus outbreaks are more frequent during cold weather months (Maalouf et al., 2010; Campos and Lees, 2014; van Beek et al., 2018; Wang et al., 2018). Enterovirus prevalence in wastewater and sewage fluctuates seasonally, with greatest levels occurring in the winter (Šimková and Červenka, 1981). Furthermore, it has been pointed out that bivalve mollusks accumulate microorganisms preferentially during periods of low temperatures, which results in a higher incidence of human gastroenteritis

acquired through shellfish consumption during these periods (Burkhardt et al., 1992; Burkhardt and Calci, 2000; Griffin et al., 2003). This preferential accumulation of microorganisms by bivalve mollusks coupled with the enhanced survival of viruses at lower temperatures, may explain the seasonality of shellfish borne viral disease (Griffin et al., 2003).

In this study norovirus contaminations have been detected in mussels collected in March, May, November and January, whereas no viral contamination has been detected in July and September showing a more frequent contamination in cold months similarly to what observe in other areas (Ilic et al., 2017; La Bella et al., 2017; Gonçalves et al., 2018).

Overall, the finding of NoV and HAV in Ravenna's harbor could represent a risk for human health because, although fishing and bathing are prohibited in the port, it must be remembered 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 activity take place. In fact, several studies have previously shown that the pathogens discharged from wastewaters pose a health risk to everyone exposed to the polluted waters, mainly during recreational users (Gonçalves et al., 2018). Actually, in the Ravenna's harbor, the presence of human virus contaminations are limited to the inner part of the harbor and to the winter period and this can greatly limit the risk for human health.

In addition to viruses of interest for human health, viruses are also important components of the marine environment and they play an enormous role in ocean process through their interactions with all types of marine organisms (Munn, 2006). Some marine viruses have been recognized as the causative agents of fish diseases (Munn, 2006; Middelboe and Brussaard, 2017) and among these the NNV, which is able to infect several finfish species (Doan et al., 2017). Accordingly, NNV is responsible of severe losses in finfish farming, but it is also responsible for damaging the natural finfish stocks (Gomez et al., 2009; Vendramin et al., 2013; Doan et al., 2017). Furthermore, NNV has been detected in several marine invertebrates, however, these species are not considered susceptible host, as viral replication has not been demonstrated in these animals. Despite this, the presence of the virus in bivalve mollusks and its surviving in these animals rise a concern about a possible role of virus carriers for these invertebrates (Volpe et al., 2017).

A previous study showed a high prevalence of NNV contamination in retail bivalve mollusks (Volpe et al., 2018). Similarly, the virological investigation conducted in the Ravenna's harbor showed a high percentage of contamination of tested mussels (30-50%) independently from the site of collection (inner sites, outer sites and offshore gas platforms). Despite some NNV susceptible species farms have been located in the surrounding lagoons (Airoldi et al., 2016), no direct water connection is present with the Ravenna harbor's area and the lagoons connected to it. Several hypotheses can explain the detection of NNV, in an area free of farms of susceptible species. The NNV could be

moved through ship transport via ballast waters, considering that Ravenna is one of the most important ports in Italy and the naval traffic is particularly intense. Numerous studies have focused on issues related to naval transport showing that a prominent vector of aquatic invasive species, pathogens and toxic species to coastal regions is the discharge of water, sediment and biofilm from ballast water tanks of ships (Drake et al., 2007; Costa et al., 2014). Several studies showed the presence of viruses in ballast water and suggest their translocation among different countries (Drake et al., 2007; Kim et al., 2016; Hwang et al., 2018). Particularly, ballast water transportation and global trade of aquatic organisms are the most common virus dispersal process identified this far (Munang'andu et al., 2016).

Genotyping of viruses detected in this study could contribute to the understanding their origin. In this study NNV detected resulted to be mainly RGNNV genotype. This is the most widespread NNV genotype in the Mediterranean Basin. Furthermore, the phylogenetic analysis showed a high similarity of the detected viruses with NNV strains previously detected in finfish and bivalve mollusks of the Adriatic Sea (Panzarin et al., 2012; Volpe et al., 2018). The reassortant strain RGNNV/SJNNV was also detected in mussels of the Ravenna's harbor. Reassortant NNV strains have been emerged by the reassortment of genotypes RGNNV and SJNNV and, so far, they have been detected only in the Mediterranean Basin (Toffolo et al., 2007; Olveira et al., 2009; Panzarin et al., 2012). These finding pointed out that NNV contaminating mussels in Ravenna's harbor seems to be autochthonous strains and suggest that these viruses could origin from sources different from ballast water.

The virus, in fact, could be directly release in this area by infected marine finfish species that normally live in this area. The 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 environmental and host factors such as temperature and high fish density, respectively. Accordingly, disease outbreaks caused by NNV are mainly described in farmed finfish during summer (Doan et al., 2017). As no susceptible finfish species farms are present in the investigated area, nor in lagoons directly connected to the harbor, we hypothesized that the NNV could be released directly by infected marine wild finfish species. NNV has been previously isolated from several asymptomatic wild marine finfish species (Ciulli et al., 2007). Furthermore, experimental trials have shown 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 of Ravenna's harbor suggests that susceptible finfish host living this area could be infected and release the virus in the water at high titers in the 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. Experimental trials demonstrate that some bivalve mollusk

species are able to accumulate and release alive viruses, including NNV (Volpe et al., 2017; Molloy et al., 2013). The accumulation and release of alive NNV by mussels in Ravenna's harbor could represent a viral source for susceptible finfish hosts, permitting to complete the epidemiological cycle of NNV infection.

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Supplementary material

Table S1

Site	Period	Date	Time (LT)	Lat	Lon	T (°C)	C (ms)	P r (mV)	pH	Ox (mg/L)	Ox (%)	S (psu)
1	May '18	04/05/2018	10:11	44,49753	12,29784	19,1	33,5	100	8,06	8,4	102,0	24,0
1	July '18	03/07/2018	10:00	44,49753	12,29784	26,8	43,1	65	8,78	6,1	83,5	26,7
1	September '18	04/09/2018	10:00	44,49753	12,29784	25,2	41,2	nd	nd	6,1	78,0	26,3
1	November '18	14/11/2018	10:40	44,49753	12,29784	16,8	25,2	91	8,30	nd	nd	18,6
1	January '19	09/01/2019	09:30	44,49753	12,29784	7,0	24,1	114	8,20	nd	nd	23,0
2	May '18	04/05/2018	11:00	44,49268	12,27859	18,5	34,2	96	8,11	8,9	106,6	24,9
2	July '18	03/07/2018	10:30	44,49268	12,27859	27,4	44,7	52	8,56	4,8	65,3	27,4
2	September '18	04/09/2018	11:10	44,49268	12,27859	25,4	41,1	nd	nd	6,8	88,9	26,1
2	November '18	06/11/2018	10:25	44,49268	12,27859	18,0	28,7	102	8,18	nd	nd	20,8
2	January '19	09/01/2019	10:30	44,49268	12,27859	7,9	25,3	108	8,48	nd	nd	23,6
5	May '18	04/05/2018	12:31	44,42893	12,22466	19,5	35,1	145	7,94	8,9	108,9	25,0
5	July '18	03/07/2018	11:00	44,42893	12,22466	27,9	32,0	18	8,76	4,9	67,7	18,7
5	September '18	04/09/2018	11:35	44,42893	12,22466	26,7	41,1	nd	nd	nd	nd	25,3
5	November '18	06/11/2018	11:05	44,42893	12,22466	18,7	35,0	111	8,09	nd	nd	25,4
5	January '19	09/01/2019	11:00	44,42893	12,22466	8,9	26,2	105	8,34	nd	nd	23,9
6	May '18	04/05/2018	13:04	44,42160	12,21020	19,9	28,0	145	7,80	7,9	92,4	19,3
6	July '18	03/07/2018	11:30	44,42160	12,21020	28,1	36,8	29	8,69	6,2	85,5	21,8
6	September '18	04/09/2018	12:10	44,42160	12,21020	27,4	42,6	nd	nd	nd	nd	26,0
6	November '18	06/11/2018	11:35	44,42160	12,21020	19,3	35,7	100	8,16	nd	nd	25,6
6	January '19	09/01/2019	04:48	44,42160	12,21020	9,0	26,5	104	8,28	nd	nd	24,1