Regional Studies in Marine Science 38 (2020) 101368

Contents lists available at ScienceDirect

Regional Studies in Marine Science

journal homepage: www.elsevier.com/locate/rsma

Environmental surveillance of human enteric viruses in wastewaters, groundwater, surface water and sediments of Campania Region

Barbara Cioffi [a,*], Marina Monini [b], Michela Salamone [c], Roberta Pellicanò [d], Ilaria Di Bartolo [b], Marco Guida [c], Giuseppina La Rosa [e], Giovanna Fusco [a]

[a] Animal Health Department, Virological Unit, Istituto Zooprofilattico Sperimentale del Mezzogiorno, Portici (NA), Italy
[b] Food Safety, Nutrition and Veterinary Health Department, Istituto Superiore di Sanità, Rome, Italy
[c] Biology Department, Università degli Studi di Napoli Federico II, Naples, Italy
[d] Regional Observatory and Biostatistic Department, Istituto Zooprofilattico Sperimentale del Mezzogiorno, Portici (NA), Italy
[e] Environment and Health Department, Istituto Superiore di Sanità, Rome, Italy

ARTICLE INFO

Article history:
Received 16 October 2019
Received in revised form 21 April 2020
Accepted 15 July 2020
Available online 31 July 2020

Keywords:
Water
Hepatitis
Environment
Rivers
Sediments
Human enteric virus

ABSTRACT

Enteric viruses represent a public health risk since they are involved in foodborne and waterborne outbreaks. The aim of this study was to investigate the circulation of select human enteric viruses — hepatitis A virus (HAV), noroviruses (NoVGI and NoVGII), adenovirus (AdV), rotavirus (RV), astrovirus (AsV), sapovirus (SaV) and aichivirus (AiV) – in wastewater and in environmental samples (groundwater, surface water and sediments) of Campania Region, southern Italy, with the goal of evaluating their circulation in the environment and to characterize the circulating strains by phylogenetic analysis. A total of 199 water and sediment samples were collected from rivers, dams, wells and wastewater treatment plants (WWTPs) in all provinces of the Campania Region and analysed by real-time qPCR for the detection of viruses, after a concentration step with organic flocculation. This study provides information on the qualitative and quantitative spread of human enteric viruses in the environment and their potential health risk. In our samples all the viral families were detected, with different prevalence. Among the investigated viruses, RV was the most prevalent and concentrated virus in surface water and sediments, with AsV being prevalent and concentrated in wastewater. The prevalence of viral pathogens was not influenced by season for almost all enteric viruses, except for NoVGII in WWTPs. Within the WWTPs, a significant reduction in the average concentration levels was observed for all viral targets from the influent to the effluent except for the HAV and AiV. The results of our study confirmed the importance of environmental surveillance that can represent a key tool for pollution control and human risk exposure.

1. Introduction

The growth of the human population and the increase of water consumption represent risk factors for the quality and availability of water resources. To address this problem, remediation and recycling strategies have been adopted to use wastewater from municipal plants for different purposes: industrial use, irrigation, recreational activities, watering and potable reuse (Kitajima et al., 2014). Water contamination with pathogens has become a global concern since the new scientific approach called "One health" has promoted the understanding that human health is closely related to environmental and animal health.

The discharge of untreated sewage is well known as the main cause of water faecal pollution, and viral waterborne diseases of man and animals are associated with the direct or indirect use of contaminated water (Fong and Lipp, 2005).

In accordance with current regulations, the assessment of water quality is based on microbiological parameters, such as *E. coli* and *Enterococcus*, which are not considered reliable indicators of the presence of viral pathogens; in fact, several studies reported the presence of enteric viruses in surface water, treated sewage and harvested mussels when bacterial indicators were not detected or were in compliance with the law (Baggi et al., 2001; Fong and Lipp, 2005; Fusco et al., 2013).

Enteric viruses, mostly non-enveloped viruses, have a high stability and persistence in the environment; in fact, they are protected against inactivation by temperature, pH and ultraviolet light. For these reasons, they can survive in a marine environment for long periods and can be carried for long distances, even several kilometres from the point of discharge, by ocean currents (Tahaei et al., 2012; Croci et al., 2000; Lees, 2000; Calgua et al.,

* Correspondence to: Istituto Zooprofilattico Sperimentale del Mezzogiorno, Via Salute 2, 80055 Portici (Na), Italy.
E-mail address: barbara.cioffi@izsmportici.it (B. Cioffi).

https://doi.org/10.1016/j.rsma.2020.101368
2352-4855/

2008). As is widely reported in the literature, enteric viruses, are known to be more resistant to common wastewater treatments than bacterial faecal indicators (Gerba et al., 2002; Bofill-Mas et al., 2006).

Viruses are excreted in the faeces of infected individuals (some enteric viruses can reach up to 10^{11} viral particles per gram of stool) (Bosch, 1998) and are introduced into the environment through the discharge of treated and untreated wastes, thereby contaminating the soil, groundwater, rivers and seawater (La Rosa et al., 2012; Okoh et al., 2010). Even if viruses are present in water at low concentrations, their infectious dose is very low, generally 1–10 viral units (Leclerc et al., 2002; Ward et al., 1984), and thus, they represent a risk to human health, even if diluted in water.

Enteric viruses replicate in the human intestine and are readily transmitted via the faecal–oral route, by direct contact, or via contaminated water, food or the environment (Koopmans and Duizer, 2004). They are responsible for many cases of nonbacterial gastroenteritis, respiratory infection, conjunctivitis and hepatitis, causing high morbidity and mortality in immunocompromised and in immunocompetent individuals worldwide (Kapikian, 2001; Lenaerts et al., 2008; Okoh et al., 2010).

Common viral families found in surface water include *Picornaviridae* (enterovirus (EV), hepatitis A virus (HAV) and aichivirus (AiV)), *Adenoviridae* (human adenovirus (HAdV)), *Caliciviridae* (norovirus (NoVs) and sapovirus (SaV)), Astroviridae (astrovirus (AsV)) and *Reoviridae* (reovirus (ReoV) and rotavirus (RV)). Within these families, norovirus, enterovirus, hepatitis A virus and adenovirus are on the Environmental Protection Agency Contaminant Candidate List (CCL), which identifies emerging contaminants that are known to occur in public water and may represent a public health risk (Kitajima and Gerba, 2015) (https://www.epa.gov/ccl).

The detection of human enteric viruses has been reported in rivers and sediments, in wastewater treatment plants (WWTP) (Calgua et al., 2013a,b; Kitajima et al., 2014; Iaconelli et al., 2017a; La Rosa et al., 2017; Farkas et al., 2018), in fresh water and recreational waters (D'Ugo et al., 2016; Wyn-Jones et al., 2011) and in mussels (Le Guyader et al., 2000; Fusco et al., 2017, 2019). They are all transmitted by the faecal–oral route and are responsible of mild or severe gastroenteritis worldwide. Characteristics of main viral water-borne pathogens are summarized in Tables 1–4 (Rusinol and Girones, 2017).

In this study, we investigated the presence, the relative abundance and genetic diversity of eight human enteric viruses in raw and treated wastewater, groundwater, surface water and sediments of the Campania Region.

The study area is approximately 512 km and includes part of the Gulf of Gaeta, the Gulf of Naples, the Gulf of Salerno and part of the Gulf of Policastro. The area is characterized by coastal cities and ports generating a urban continuum (approximately 6.4%), outputs of polluted rivers, lack of adequate WWTP along the entire Region, industrial districts (approximately 1.32%) and agricultural sites (approximately 65.3%) (ISPRA AMBIENTE CLC http://www.isprambiente.gov.it/it/temi/suolo-e-territorio/copertura-del-suolo/corine-land-cover). However the whole Region is an international attraction for tourism for its historical and natural sites, and its coastline is notorious for leisure activities, fishery, mussel farming and cruise income (Tornero and Ribera d'Alcalà, 2014). The above described anthropic pressure results in an increased water pollution that can represent a risk for human health, in fact, several gastrointestinal outbreaks are caused by bathing in contaminated waters (Tosone et al., 2016) and by the consumption of raw or undercooked mussels harvested in faecal polluted waters (Fong and Lipp, 2005).

The goal of our study was to evaluate the presence and the relative abundance in the water environment of human enteric viruses and to characterize the circulating strains by phylogenetic analysis.

We tested our samples for the presence of hepatitis A virus, norovirus genogroups I and II (NoVGI, NoVGII), human adenovirus, rotavirus, astrovirus, sapovirus and aichivirus by real-time qPCR after the concentration step, in WWTP, groundwater, surface water and sediments over one year.

2. Material and methods

2.1. Sample collection

From July 2017 to August 2018, 199 water and sediment samples were collected from different environmental matrices and WWTP in all provinces of the Campania Region. Of these, 95 samples were groundwater, surface water and sediments and 104 were wastewater. Within the environmental samples (groundwater, surface water and sediments), 43 were river water (10 l) sampled together with 40 river sediments (1 g of the top layer), 10 were groundwater from wells located in farmhouses, and 2 were dam water (10 l). The water and sediments were sampled, every six months, from the main rivers of the Campania Region: the Volturno and Regi Lagni in the province of Caserta; the Sele, Irno, Bussento, Picentino, Tanagro and Tusciano in the province of Salerno; and the Calore and Sarno rivers, sampled in sites in more than one province. In addition, water samples from dams located on the Ufita and Alento rivers, which serve as important sources of water for local agriculture, and groundwater from farm wells, used for irrigation and watering, were included in our campaign. A sampling scheme is summarized in Fig. 1.

Wastewater was collected from five major WWTP, every 4 months, located in each Province of the Campania Region and from twelve minor plants, every 6 months, located in municipalities mainly with a rural or touristic vocation. The major five WWTP treat sewage from different-sized communities and from urban, industrial and rural areas. In each major plant (>100,000 equivalent inhabitants), samples were collected at five different treatment points: influent (50 ml each), sedimentation tank I (50 ml each), sedimentation tank II (50 ml each), sludge (1 g each) and effluent (10 l each). In the twelve minor plants (500–50,000 equivalent inhabitants), only the influent was sampled (10 l each). Wastewater in the WWTP is mainly processed by biological treatment except for the WWTP located in Naples (San Giovanni) that has a chemical–physical treatment process. The geographic locations of the rivers, the WWTP and the study area are shown in Fig. 2.

Water samples were collected in sterile plastic tanks, and the sediments of all river samples and sludge from the WWTP were aseptically collected with a bucket and stored in sterile glass. All samples were transported to the laboratory and stored for a maximum of 24 h at 4 °C before being processed.

2.2. Concentration of samples

When investigating viruses in environmental water, large sample volumes are required, since these viruses are present at low concentrations, but even a few particles from these viruses can be infective for humans. Therefore, large water sample volumes guarantee an efficient recovery of pathogens. The sample volume depends on the degree to which the water is faecally contaminated; for rivers, irrigation water, seawater and groundwater, several litres of sample are needed to concentrate the viruses, whereas, for contaminated waters such as sewage or wastewater, smaller volumes can be processed to achieve viral recovery. In particular, Calgua et al. (2008, 2013b) reported a one-step concentration procedure based on organic flocculation with skimmed milk to recover pathogens in water samples of different volumes.

Table 1
Summary of the characteristics of main viral water-borne pathogens indicating the Family, Genus and Species, transmission routes, seasonality and related disease.

Family (genome, size)	Genus	Most important human pathogens	Related diseases	Transmission routes	Seasonality
Adenoviridae (dsDNA, 70–90 nm)	Mastadenovirus	Human adenovirus A–G (HAdV)	Gastroenteritis, respiratory disease, conjunctivitis, cystitis	faecal–oral: contaminated food, person-to-person, drinking water Airborne: respiratory secretions Bathing water	Without clear seasonality
Astroviridae (ssRNA, 28–41 nm)	Mamastrovirus	Astrovirus 1–9 (AsV)	Gastroenteritis, related to respiratory infections	faecal–oral: contaminated food, person-to-person, drinking water	Higher prevalence in the cold-weather period
Caliciviridae (ssRNA, 27–38 nm)	Norovirus	Norovirus GI, GII (NoVs)	Gastroenteritis	faecal–oral: contaminated food, person-to-person, drinking water	Higher prevalence in cold months
	Sapovirus	Sapovirus GI, GII, GIV, GV (SaV)	Gastroenteritis	faecal–oral: drinking water	Peak observed mainly in the cold season
Picornaviridae (ssRNA, 24–30 nm)	Hepatovirus	Hepatitis A virus GI–III (HAV)	Acute hepatitis	faecal–oral: contaminated food, person-to-person	No clear seasonality
	Kobuvirus	Aichivirus A to C (AiV)	Gastroenteritis	faecal–oral: contaminated food, person-to-person, drinking water	Without clear seasonality
Reoviridae (dsRNA, 70–75 nm)	Rotavirus	Rotavirus A to G (RV)	Gastroenteritis	faecal–oral: contaminated food, person-to-person, drinking water	Year-round infection in countries within 10 degrees of the equator; Winter peaks in all other regions of the world

Data from Global Water Pathogen Project, UNESCO (http://www.waterpathogens.org/book/summary-of-excreted-and-waterborne-viruses).

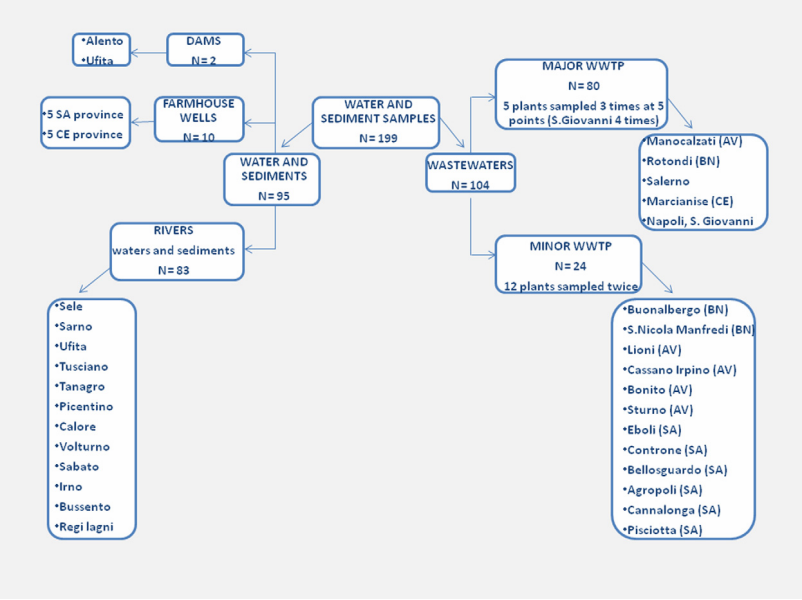

Fig. 1. Graphic scheme of collection sites – describing the zone or name – and sample types — describing the nature of samples.

2.2.1. Murine norovirus process control

Before concentration a murine norovirus (MNV) was added to all samples as a sample process control to calculate viral recovery and evaluate potential inhibition as previously described (Iaconelli et al., 2015). In particular, Murine macrophage-like RAW264.7 cells grown in Dulbecco's Modified Eagle's Medium (D-MEM) were inoculated with MNV-1 (MNV-IT1 Acc. no. KR349276). Three days after the inoculation, the solution was removed. Then, every 3 days, the medium was replaced and used to infect new RAW264.7 cell monolayer. After six passages, the viral titre was calculated by end-point dilution and a final stock of 10^7 PFU ml^{-1} was obtained and used to spike the samples.

2.2.2. Water samples

Before concentration, 1 ml MNV was added to all samples.

Ten litres of water samples were concentrated by skimmed milk flocculation (SM), as previously described by Calgua et al.

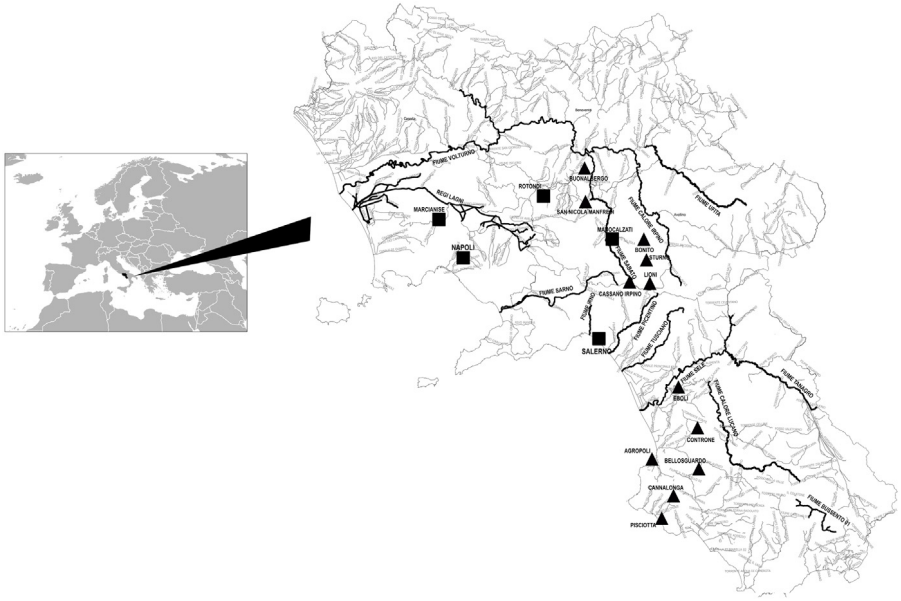

Fig. 2. Map of the study area with location of rivers and WWTP in the Campania Region: black lines are rivers, ■ major plants treating sewage of big communities (>100,000 equivalent inhabitants), ▲ minor plants treating sewage of small–medium communities (500–50,000 equivalent inhabitants).

(2013b) and Bofill-Mas et al. (2011). Briefly, after conditioning, a pre-flocculated 1% (w/v) skimmed milk solution (PSM) was prepared by dissolving 10 g of skimmed milk (Difco, Detroit, MI, USA) in 1 l of artificial seawater at pH 3.5 (Sigma, Aldrich Chemie GMBH, Steinheim, Germany). The sample was then acidified to pH 3.5 by adding HCl 1 N along with the PSM until a final concentration of 0.01% (w/v) of skimmed milk in the sample was reached (100 ml of PSM 1% in 10 l of sample). Samples were stirred for 8 h at room temperature and allowed to rest for another 8 h to let the flocks form sediment by gravity; then, the supernatant was carefully removed using a vacuum pump. The concentrated sample of approximately 500 ml, which contained sedimented flocks, was transferred to a centrifuge tube and centrifuged at 8000 xg for 30 min at 4 °C. The supernatant was carefully removed, and the pellet was dissolved in phosphate buffer saline (PBS), (1:2, v/v) at a ratio of 1 ml of PBS per litre of sample. The concentrated sample was stored at −80 °C or processed for extraction of the viral nucleic acid.

2.2.3. Wastewater samples

Wastewater samples were concentrated by SM, as previously described by Calgua et al. (2013a). Briefly, the sample (50 ml) was transferred to a 500 ml centrifuge pot, and 100 ml of glycine buffer 0.25 N, pH 9.5 (1:2, v/v) was added. The sample was stirred rapidly for 30 min on ice and centrifuged at 8000 ×*g* for 30 min at 4 °C. The supernatant (150 ml) was transferred to a new centrifuge pot, the pH was adjusted to 3.5 with HCl 1 N, and 1.5 ml of PSM was added until a final concentration of skimmed-milk 0.01%, (w/v) in the sample was reached (1.5 ml of PSM 1% in 150 ml of sample). The PSM solution was prepared as described in Section 2.2.2. At this step, a 10 μl MNV was added to all samples. The sample was then stirred for 8 h at room temperature and allowed to rest for another 8 h to allow the flocks to form sediment by gravity. Then, the flocks were sedimented by centrifugation at 8000 ×*g* for 30 min at 4 °C, the supernatants were carefully removed, and the pellet was dissolved in 500 μl of phosphate buffer saline.

2.2.4. Sediment samples

Before concentration, 10 μl MNV had been added to all samples. The river sediment underwent a direct extraction of viral nucleic acids by adding modified Eagle's Minimal Essential Medium (E-MEM), as previously described by Staggemeier et al. (2015). Briefly, 1 g of sediment and 1 ml of E-MEM were mixed, and the pH was adjusted to 11.5 by adding NaOH 1 M or HCl 1 N. The solution was vortexed for 1 min and then centrifuged at 10,000 × *g* for 10 min. The supernatant was then used for the extraction of the viral nucleic acid.

2.3. Viral nucleic acid extraction

Nucleic acids were extracted from 400 μl of concentrated samples using the QIAsymphony (Qiagen, Hilden, Germany) automated system based on magnetic silica purification. DSP Virus/Pathogen cartridges and protocols were used according to the manufacturer's instructions. Eluates (80 μl) were stored at −80 °C or used for downstream analysis.

2.4. Real-time quantitative polymerase chain reaction

Real-time quantitative polymerase chain reaction (qPCR) was performed on a 7500 Fast Real-Time PCR thermocycler (Applied Biosystems, Foster City, California, USA). The RNA UltraSense reaction kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and Express qPCR SuperMix (Invitrogen, Carlsbad, California, USA) were used for RNA and DNA viruses, respectively, in final reaction volumes of 25 μl, with 5 μl of DNA/RNA extract as the target. Each viral target was analysed, both undiluted and diluted 10^{-1}, in duplicate with primers (Tema Ricerca, BO, Italy) and a TaqMan fluorogenic probe (Thermo Fisher Scientific, Waltham, Massachusetts, USA) specific for the virus of interest, as listed in Table 2. Positive, no-template and inhibition controls were included in each run. HAV, NoVGI and NoVGII positive controls were plasmids kindly provided by the Italian National Reference Laboratory for Monitoring of Viral Contamination of Bivalve Molluscs (Istituto Superiore di Sanità, Rome, Italy); HAdV and RV plasmids were kindly provided by the Laboratory of Food Safety, Nutrition and Public Veterinary Health (Istituto Superiore di Sanità, Rome, Italy); AsV and SaV Q standards were purchased from Ceeram Tools (Biomerieux); a human stool sample positive to AiV was kindly provided by the Reference des Virus Enteriques (Dijon, Cedex, France). PCR inhibition was evaluated using an external amplification control.

Table 2
Primers and probes used for real-time qPCR for each target virus.

Primers and probes	Sequence (5′-3′)	Reference
HAV		
HAV68 (Fw)	TCACCGCCGTTTGCCTAG	Costafreda et al. (2006)
HAV240 (Rev)	GGAGAGCCCTGGAAGAAAG	
HAV150 (Probe)	FAM-CCTGAACCTGCAGGAATTAA-MGBNFQ	
NG I		
QNIF4 (Fw)	CGCTGGATGCGNTTCCAT	da Silva et al. (2007) Svraka et al. (2007)
NV1LCR (Rev)	CCTTAGACGCCATCATCATTTAC	
NVGG1p (Probe)	FAM-TGGACAGGAGAYCGCRATCT-TAMRA	
NG II		
QNIF2 (Fw)	ATGTTCAGRTGGATGAGRTTCTCWGA	Loisy et al. (2005) Kageyama et al. (2003)
COG2R (Rev)	TCGACGCCATCTTCATTCACA	
QNIFs (Probe)	FAM-AGCACGTGGGAGGGCGATCG-TAMRA	
RV		
NSP3F (Fw)	ACCATCTWCACRTRACCCTCTATGAG	Zeng et al. (2008)
NSP3R (Rev)	GGTCACATAACGCCCCTATAGC	
NSP3P (Probe)	FAM-AGTTAAAAGCTAACACTGTCAAA-MGB	
AsV		
AV1 (Fw)	CCGAGTAGGATCGAGGGT	Le Cann et al. (2004)
AV2 (Rev)	GCTTCTGATTAAATCAATTTTAA	
AVs (Probe)	FAM-CTTTTCTGTCTCTGTTTAGATTATTTTAATCACC-TAMRA	
SaV		
SAV124F (Fw)	GAYCASGCTCTCGCYACCTAC	Varela et al. (2015)
SAV1245R (Rev)	CCCTCCATYTCAAACACTA	
SAV124TP (Probe)	FAM-CCCCTATRAACCA-NFQ MGB	
AIV		
AiV-AB-F (Fw)	GTCTCCACHGACACYAAYTGGAC	Kitajima et al. (2013)
AiV-AB-R (Rev)	GTCTCCACHGACACYAAYTGGAC	
AiV-AB (Probe)	FAM-TTYTCCTTYGTGCGTGC-NFQ MGB	
HAdV		
AdF (Fw)	CWTACATGCACATCKCSGG	Hernroth et al. (2002)
AdR (Rev)	CRCGGGCRAAYTGCACCAG	
AdP1 (Probe)	CCGGGCTCAGGTACTCCGAGGCGTCCT- BHQ1	
MuNoV		
MNoV F (Fw)	CACGCCACCGATCTGTTCTG	Baert et al. (2008)
MNoV R (Rev)	GCGCTGCGCCATCACTC	
MGB-ORF1/ORF2 (Probe)	CGCTTTGGAACAATG-MGB-NFQ	

Quantification was made by calibration curves generated by amplifying tenfold serial dilutions of a standard plasmid cDNA containing the target sequences (from 1 to 1×10^6 genome copies per reaction) specific for each viral target. Log genome copies were plotted against the CT number, and the results were expressed as the number of genome copies per gram of sediment or per litre of water (copies g^{-1} or copies l^{-1}).

The amplification efficiency (E) for each standard curve was calculated as previously described by Amoroso et al. (2011).

The viral recovery efficiency for MNV was determined as previously described by Iaconelli et al. (2015), as the ratio between the number of genome copies (gc) recovered for each sample after concentration and the gc of the viral stock used to spike the samples.

2.5. Sequencing and phylogenetic analysis

All positive samples exhibiting a threshold cycle $\leq$37 underwent sequence analysis. The other samples were considered positive but were not concentrated enough to allow sequencing analysis.

RNA samples were subjected to RT-PCRs using the OneStep RT-PCR kit (Qiagen, Hilden, Germany), which was followed by nested or semi-nested PCR using GoTaq Master Mix (Promega, MI, Italy). HAV sequencing was performed with a nested PCR with degenerated primers able to amplify all HAV genotypes (Taffon et al., 2011). For rotavirus, the obtained RNA was used as a template for PCR amplification of VP7 and VP4 gene segments. RVA genotyping was carried out by a multiplex semi-nested PCR using a mixture of primers specific for G- and P-types, as previously described (Gentsch et al., 1992; Gouvea et al., 1990). RNA from samples positive for norovirus was analysed by RT-PCR, with primer sets G1SKF/G1SKR and G2SKF/G2SKR annealing to ORF2 (Kojima et al., 2002) and specific for the GI and GII genogroups, respectively. For SaV genotyping, RT-PCR was used to amplify a region of 432 bp in the ORF1–ORF1 junction (Yan et al., 2003), followed by two semi-nested PCRs specific for each human genotype (Monica et al., 2007). Aichivirus sequencing was performed by RT-PCR that amplified a 730 bp fragment in the VP3 and VP1 junction, followed by two different nested-PCRs that amplified 530 bp and 264 bp fragments in the VP1 (Lodder et al., 2013). For AsV, the RT-PCR was carried out using primers that targeted the ORF2 by amplifying a 449 bp fragment (Noel et al., 1995), and finally HAdV was sequenced by an RT-PCR followed by nested PCR that amplified 301 and 171 bp fragments in the hexon, respectively (Allard et al., 2001; Pina et al., 1998).

Amplicons obtained by RT-PCR or nested-PCR of the expected sizes were purified using Exonuclease I and shrimp alkaline phosphatase (ExoSAP-IT, Affymetrix, USB) or QIAquick PCR Purification Kit (Qiagen, Hilden, Germany).

Sequencing of the DNA fragments was performed by Eurofins (Italy), with the same primers used for the amplification. The resultant sequences were analysed and corrected with ChromasPro 2.23 software (Technelysium, Queensland, Australia), aligned with known sequences retrieved from GenBank NCBI, and analysed for strain genotype identification and for comparison with known sequences. Norovirus genotypes and variants were assigned using the public database NoroNet typing tool (http://www.rivm.nl/mpf/norovirus/typingtool).

Sequences of the viral strains identified in this study were deposited in GenBank, under the following accession numbers: NoV MK039439, RV MK039440-42, AiV MK041035 and AstV MK041036-38.

2.6. Statistical analysis

Statistical analyses on results were performed using IBM SPSS Statistics 24 software, Statistical Analysis (IBM Corp., Armonk, NY, USA).

A 2 X 2 contingency table and Fisher's exact Test was applied on qualitative data (presence/absence) to focus on the influence of seasonality on the presence of enteric viruses. The significance threshold was set to $p = 0.05$. The non-parametric Mann–Whitney test for non-paired data was used to compare quantitative data of viral load from the inlet to the output of the WWTP. The significance threshold was set to $p = 0.05$. A Pearson's Chi-squared test, with one degree of freedom, was performed on data obtained from environmental samples (waters and sediments) to evaluate correlations among viruses. P values <0.05 were considered statistically significant.

3. Results

3.1. Virus detection by PCR

A total of 199 samples (104 wastewater, 43 river water, 40 river sediment, 10 well water and 2 dam water samples) were collected and analysed for the qualitative and quantitative detection of enteric viruses by real-time qPCR. The results are summarized in Table 4.

All the selected samples in this study achieved acceptable recovery rates (>5%). Recovery rates of all samples ranged between 5 and 85%, and the average value was 30.6%.

3.1.1. Water and sediments

Considering overall viral detection, the results show that 61% of the samples (58/95) were contaminated by at least one of the investigated viruses. The percentage of contaminated samples increased to 69.7% (30/43) in river water and decreased to 57.5% (23/40) in river sediments, to 50% (1/2) in dam water and to 40% (4/10) in well water.

Among the 58 positive samples, the simultaneous presence of several viral families (up to four) was observed in 31% (18/58) of samples while 69% (40/58) of the samples were contaminated with a single family of virus. Within the positive samples, 51.7% (30/58) were represented in river water, 39.6% (23/58) in rivers sediments, 6.9% (4/58) in farmhouse well water and 1.7% (1/58) in dam water.

During our study, the most frequently detected virus was RV (53.4%). The other viruses were AsV (34.5%), SaV (22.4%) and NoVs (13.8%, for NoVGI and 8.6%, for NoVGII). AiV was found in 4 samples (6.9%), and HAV RNA was identified in 2 (3.5%) river samples, from the Ufita and Sele rivers. HAdV was detected only in 1 (1.72%) river sample, from the Tusciano River (Battipaglia).

HAV, NoVs and HAdV were detected only in river waters, whereas, in river sediments, the revealed targets were RV, SaV, AsV, and AiV.

For these substrates, a correlation among the tested viruses was evaluated using a Pearson's Chi-squared test. The statistical analyses revealed a significant ($p<0.01$) correlation among NoVGI and NoVGII, among AsV, AiV and NoVGI, and among NoVGI, NoVGII and HAdV (Table 3).

3.1.2. Wastewater

In wastewater samples, higher abundance and viral concentrations were observed compared to groundwater, surface water and sediments. In fact, the results of viral detection in the wastewater show that 92% of the samples (96/104) were contaminated by at least one of the investigated viruses. Among the 96 positive samples, the simultaneous presence of several viral families (up to eight) was observed in 86% (83/96) of the samples, whereas only 13% (13/96) was contaminated with a single family of virus. The percentage of samples found positive at the different collection points of the WWTP was 100% for the influents; 94% and 88% for sedimentation tanks I and II, respectively, 100% for the sludge and 73% for the effluents.

The most frequently detected virus in the WWTP was AsV, with a prevalence of 74% (71/96). The other viruses were SaV (57%), RV (55%), NoVs (50% for NoVGII and 46% for NoVGI), AiV (40%) and HAdV 29%. HAV RNA was identified in 12 (12.5%) wastewater samples collected in all points of the WWTP. The presence of all the investigated viruses was reported in each of the five collection points of the WWTP. AsV was the most prevalent virus in the influent, in the I and II sedimentation tanks and in the effluent, whereas RV was the most prevalent virus in the sludge. The results for all substrates are summarized in Table 4.

3.2. Standard curves and virus quantification

The efficiencies of amplification ($E = 10^{-1/S} - 1$, where S is the slope of the linear regression curve) for each standard curve were as follows: $E_{HAV} = 1.16$ ($S = -2.98$, $r^2 = 0.99$), $E_{NoVGI} = 1.08$ ($S = -3.15$, $r^2 = 0.99$), $E_{NoVGII} = 1.18$ ($S = -2.95$, $r^2 = 0.99$), $E_{RV} = 1.13$ ($S = -3.04$, $r^2 = 0.99$), $E_{HAdV} = 1.0$ ($S = -3.30$, $r^2 = 0.99$), $E_{AsV} = 0.95$ ($S = -3.43$, $r^2 = 0.99$), $E_{SaV} = 0.96$ ($S = -3.43$, $r^2 = 0.99$), and $E_{AiV} = 1.0$ ($S = -3.27$, $r^2 = 0.99$). The teoric limit of detection for each target virus was 5 GC/reaction.

3.2.1. Water and sediments

In the quantitative analyses, most of the viruses show concentrations ranging between 10^1 and 10^4 gc per gram or per litre; only RV was found at higher levels, reaching 10^5 gc in the river water and sediments, whereas HAdV was detected only in one river sample at a concentration under the LOD. RV yielded the highest average concentration of 7.4×10^4 gc l^{-1} of water and 4.3×10^4 gc g^{-1} of sediment. SaV and AsV were detected in the river water, with an average concentration of 1.3×10^4 gc l^{-1} and 6.8×10^3 genome copies l^{-1}, respectively, and in sediments with 1.5×10^2gc g^{-1} and 2.6×10^2 gc g^{-1}, respectively. AiV was found in two river samples at an average concentration of 1.8×10^3genome copies l^{-1} in water and 1.0×10^3 gc g^{-1} in sediments. The NoVs showed an average concentration of 2.3×10^2 gc l^{-1} for NoVGI and 1×10^4 gc l^{-1} for NoVGII in river water, but these viruses were not found in sediments. HAV RNA was recovered from 2 river water samples, taken from the Ufita and Sele rivers, at respective concentrations of 1.0×10^2 and 2.2×10^1 gc l^{-1}. Quantitative results of viral load in environmental samples (waters and sediments) are shown in Fig. 3.

3.2.2. Wastewater

Viral concentrations in wastewater were 1–3 $\log_{10}$ higher than that observed in water and sediments. In fact, in wastewater, the quantitative analyses showed concentrations ranging between 10^1 and 10^7 genome copies per litre; only AsV reached the maximum concentration of 10^8 gc l^{-1} in the sedimentation tank I collection point. AsV resulted the most concentrated virus in each point of collection, except in sludge, reaching the average value of 1.7×10^7gc l^{-1} in the sedimentation tank I. AsV, RV and AiV

Table 3
Correlation among viruses. Statistical analysis was carried out on environmental samples (waters and sediment) by Chi-squared test and the correlations considered statistically significant ($p < 0.01$) are in indicated in grey (N = 95).

	HAV	NoVGI	NoVGII	RV	HAdV	AsV	SaV	AiV
HAV	1	-0.044	-0.035	-0.102	-0.015	-0.076	0.155	-0.032
NoVGI		1	0.268**	0.031	0.340**	0.401**	0.100	0.123
NoVGII			1	0.037	0.438**	0.110	0.043	-0.052
RV				1	-0.072	-0.139	-0.146	-0.143
HAdV					1	-0.053	-0.041	-0.023
AsV						1	0.170	0.275**
SaV							1	-0.088
AiV								1

**. Correlation is significant for p<0.01 (two tail).

Table 4
Number of real-time PCR positive samples.

n. multiple positive samples/n. total samples	Matrix	HAV	NoVGI	NoVGII	HAdV	RV	AsV	SaV	AiV
35/55 (63%)	Water	2	8	5	1	14	17	9	2
23/40 (57.5%)	Sediments	0	0	0	0	17	3	4	2
96/104 (92%)	Wastewater	12	44	48	28	53	71	55	38
40/40 (100%)	*Influent*	5	20	28	15	22	34	27	20
15/16 (93.8%)	*Sedimentation I*	3	9	12	7	6	13	11	6
14/16 (87.5%)	*Sedimentation II*	1	6	3	1	5	9	7	5
16/16 (100%)	*Sludge*	2	3	2	2	15	6	5	6
11/16 (69%)	*Effluent*	1	6	3	3	5	9	5	1

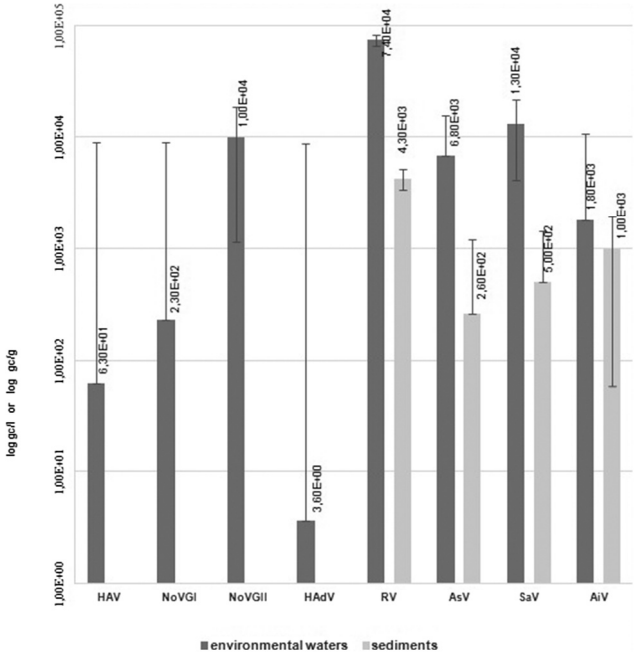

Fig. 3. Arithmetic mean concentration of viral targets determined by real-time qPCR in environmental samples (waters (n = 55) and sediments (n = 40)). Error bars represent standard deviation.

showed comparable abundance in the influent, 7.4×10^6, 1.2×10^6 and 3.4×10^6gc l^{-1}, and 3×10^4, 1.8×10^4 and 1.6×10^4 gc l^{-1} in the effluent, respectively. SaV and HAdV showed concentrations of 1×10^6 and 5.4×10^6gc l^{-1} in the influent and 6.5×10^2 and 2.9×10^3 gc l^{-1} in the effluent, respectively. NGI and NGI were detected, respectively, at an average concentration of 3.2×10^5 and 1.3×10^5 gc l^{-1} in the influents and at 1.6×10^2 and 1.2×10^2 gc l^{-1} in the effluents. Hepatitis A virus was detected at lower concentrations of 2.1×10^3 gc l^{-1} in the influent and 1.5×10^2gc l^{-1} in the effluent. The virus reduction from the influent to the effluent in WWTP was evaluated with a non-parametric Mann–Whitney test for non-paired data, which showed that the decrease was statistically significant (p<0.05) for all viruses except for AIV ($p = 0.121$) and HAV ($p = 0.143$). Even if an average reduction of 3 $\log_{10}$ in the viral loads was observed during the depuration process, the quantity of the investigated viruses remained high in the effluent, showing values ranging from 1.5×10^2 for HAV to 3×10^4gc l^{-1} for AsV. Quantitative results of viral load in wastewater are shown in Fig. 4.

3.3. Phylogenetic analysis of detected viruses

Amplicons derived from positives samples were further characterized by sequencing.

Three HAV sequences were obtained from wastewater samples within the same WWTP of Naples: from the sedimentation tank I, from the effluent and from the sludge. They grouped into two different clusters, according to BLAST analysis: genotype IB and IA (Fig. 5). One NoV GII strain was confirmed by sequencing and was assigned to the GII.P17 genotype. Three sequences obtained from the RVA positive samples showed an equine-like VP7 gene G3 genotype. One Aichi virus 1 strain (AiV-1) was identified as belonging to genotype A. For AsV, three samples were confirmed by sequencing, and they were classified as human Mamastrovirus genotype 1.

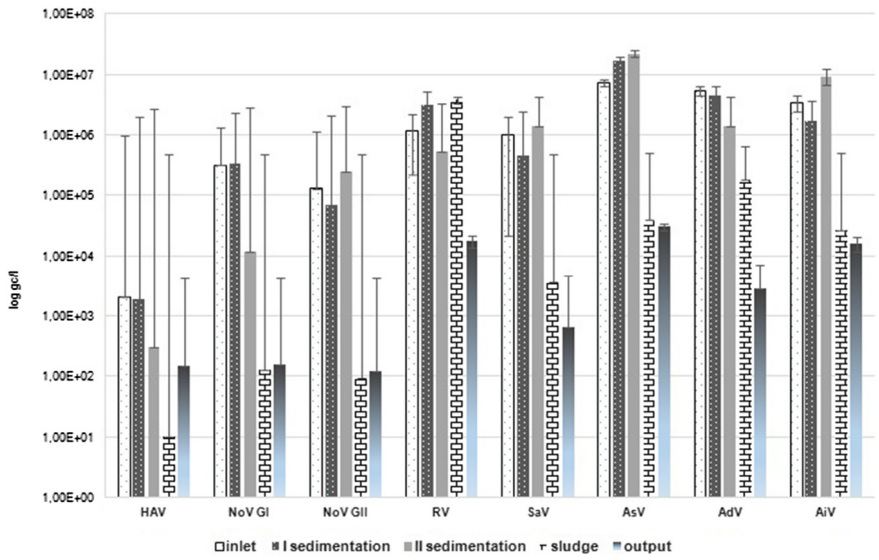

Fig. 4. Arithmetic mean concentration of viral targets determined by real-time qPCR in wastewater (n=104) samples. For each target the viral load has been reported for the inlet (n=40), I sedimentation (n=16), II sedimentation (n=16) and the output (n=16). Error bars represent standard deviation.

3.4. Seasonality of enteric viruses

The seasonal distribution of viral pathogens was evaluated during winter/autumn months (from November to April) and spring-summer months (from May to October) with a statistical test for each viral population (Fisher's exact Test on qualitative data with $p < 0.05$ considered as significant). The prevalence of viral pathogens was not influenced by season for almost all enteric viruses, except for NoVGII in WWTP, whose presence is influenced by seasonality ($p = 0.039$), according to Fisher's exact Test.

4. Discussion

In this study, we provided information on the environmental contamination of conventional and possible emerging human enteric viruses (HAV, NoVGI, NoVGII, HAdV, RV, AsV, SaV and AiV) in 199 wastewater and environmental samples (groundwater, surface water and sediments) in the Campania Region to evaluate their circulation and spread in the environment, since they can impact on regional coastal water quality.

To our knowledge, this is the first study on the occurrence of a large panel of human enteric viruses in environmental samples in Campania Region. In our study, all viruses included in the panel were identified at different frequencies and concentrations in all the analysed matrices, except for HAdV, which was detected only in wastewater and in one river water sample.

As expected, and as previously reported in other European studies (Farkas et al. 2018), detection frequencies and concentrations of all target viruses were significantly lower in water and sediments than in wastewater in our surveillance study. Within the WWTP, a statistically significant reduction in the average concentration levels was observed for all viral targets from the influent to the effluent as described in other papers (Kitajima et al., 2014; Iaconelli et al., 2017a) except for the HAV and AiV. However, the quantity of the investigated viruses remains high in the effluent, confirming that common depuration processes are not efficient in viral removal and that improperly treated effluents represent one of the main vehicles of virus dissemination in the environment (Battistone et al., 2014; Iaconelli et al., 2017a).

Analysing data of environmental samples (waters and sediments), a possible correlation among the detected viruses was also evaluated, since samples frequently resulted positive to more than one virus. Correlations between the presence of NoVGI and NoV GII were observed. The occurrence of NoVGI and NoVGII was strictly correlated with the presence of HAdV, and a further correlation was observed between AsV, AiV and NoVGI. Further studies are needed to evaluate whether a quantitative correlation also exists.

The incidence of HAV infection reaches the highest rate in non-industrialized countries, where wastewater treatment and hygiene conditions can be scarce (Tahaei et al., 2012). The epidemiology of HAV in Italy, considered as a medium–low endemicity country, recorded epidemics associated with the consumption of raw seafood in 1992, 1994 and 1997, and, in some regions of southern Italy, a new epidemic of HAV was linked to the consumption of frozen berries in 2013 (Costantino et al., 2017). In 2016 and 2017, an excess of cases of HAV occurred in 13 European countries, including Italy, and were associated with three different clusters of infection, predominantly affecting men who have sex with men (MSM) in South Italy (Mauro et al., 2019); however, water is still considered one of the main vehicles of transmission of HAV (Rodríguez-Lázaro et al., 2012).

In our study, the presence of HAV RNA was detected in river samples from the Ufita and Sele rivers, and in wastewater (influents and effluents). Unfortunately, probably due to their low concentrations, characterization analysis by sequencing was not achieved for the river water samples, but HAV strains with genotype IA and IB have been identified in wastewater samples and mussels from the same Region (Fusco et al., 2019). In particular, in two wastewater samples (sludge and effluent), the isolated strains with genotype IA showed a 99% nucleotide (nt) identity with strains circulating in Italy and Europe, at the end of 2016 (Accession numbers KY886891, KY292290, KY782330), reported in the MSM outbreak, as shown in Fig. 5 (Fusco et al., 2019).

The presence of HAV already has been reported in the Tiber River (La Rosa et al., 2017), in the Po River and in wastewater (Iaconelli et al., 2015) and mussels in the Campania Region (Fusco et al., 2017). Moreover, a case report by Tosone et al. (2016) described an HAV outbreak in a family cluster, identified as an uncommon genotype IA, not related to common risky foods but to Sele river water, which the family had used recreationally. These data underline the role of water in the spread of this pathogenic virus and the likelihood that it will infect humans,

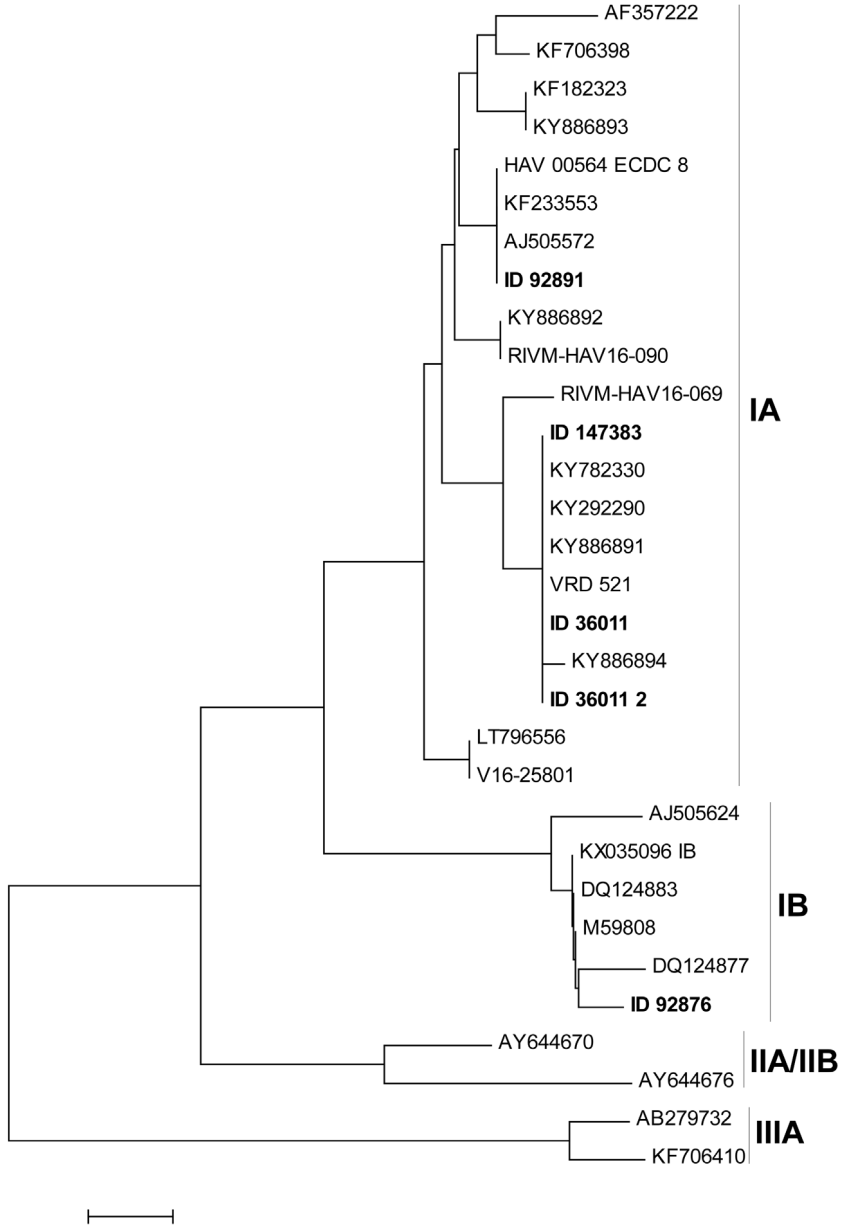

Fig. 5. Phylogenetic tree displaying the genetic relationship between environmental (in bold) and GenBank HAV isolates. ID 36011 and 36011-2 are mussel samples; ID 92876, 92891 and 147383 are wastewater samples.

even at low concentrations, due to its low infectious dose and persistence in the environment (Iaconelli et al., 2015). Despite hepatitis A being subject to notification, the circulation of HAV in the Campania Region is underestimated, as many human cases linked to the consumption of raw seafood and may not have required hospitalization.

Noroviruses have been detected in municipal waters, sewage, rivers and sediments, recreational water, surface and groundwater worldwide (La Rosa et al., 2012; D'Ugo et al., 2016; Farkas et al., 2018), and several waterborne outbreaks have been described in the literature (Hoebe et al., 2004; Kvitsand and Fiksdal, 2010; Nenonen et al., 2012). Unlike other studies, we detected NoVs in wastewater and river water but not in sediments (Farkas et al., 2018). In river water, the prevalence of the NoVs was comparable to that reported by Farkas et al. (2018) in river waters and by Wyn-Jones et al. (2011) in recreational waters, but the results were lower than those of other European rivers (Calgua et al., 2013b; Iaconelli et al., 2017a; Prevost et al., 2015). The higher prevalence of NGI in the river water samples, already reported in the literature, may be linked to a probable different stability in the water environment of the two genotypes (Myrmel et al., 2015). In wastewater, both NoVGI and NoVGII were detected at similar frequencies comparable to that reported by Iaconelli et al. (2017a). The wide variability of prevalence found in the different European studies is probably related to the seasonal spread of these viruses, which are usually detected with higher frequencies and concentrations in cold waters (Myrmel et al., 2015; Wyn-Jones et al., 2011; Prevost et al., 2015). Unlike other studies, the presence, in our samples, of NoVGII in WWTP showed a significant higher prevalence in the warm season. However, it is known that the virus concentration in WWTP may vary widely due to the type of treatment process, season, location, and hygiene conditions of population, which makes it difficult to predict the occurrence of viral pathogens in wastewater.

In our study, NoVs sequenced strains were assigned to GII.P17. The novel GII.P17 was first reported in Asia in 2014, and later on,

it was detected in Europe, including hospitalized children in Italy and in seawater discharges and bivalve shellfish (da Silva et al., 2007; Medici et al., 2015; La Rosa et al., 2017). The novelty of this genotype emerged and replaced the pandemic strain GII.4 Sydney 2012. The results confirm, although for only one sample, the importance of viral detection in water samples for surveillance of human norovirus cases.

Rotavirus was the most prevalent and abundant virus in environmental samples (water and sediments), reaching a concentration of 10^5 genome copies (per litre and per gram) in both matrices; its prevalence in wastewater was comparable to that of river water, but its concentration in wastewater was higher. The wide spread of RV in the environment could be explained both by its high excretion rate in the population and by the nature of its genome – double stranded RNA – that results in more resistance to diffused endonuclease activity (Espinosa et al., 2008). This virus is considered the main viral agent responsible for diarrhoeal disease in children worldwide, and in fact, its presence has been described in other countries in treated and untreated wastewater and surface water (Prevost et al., 2015; Grassi et al., 2010). However, such a high prevalence in surface water rarely has been reported in the literature, where its presence has been reported with lower detection frequencies (La Rosa et al., 2017; Calgua et al., 2013b). In this study, sequencing analyses identified the circulating RV strains as genotype A G3, which has the broadest host range, having been detected in equine and many other animal species (canine, feline, ovine, porcine, and murine), thus confirming the importance of environmental surveillance in detecting viral pathogens circulating in human and animal populations.

So far, HAdVs have been proposed as a viral indicator of faecal water pollution because of their high prevalence in the population and high stability in the environment, which is probably due to their double-stranded DNA (Iaconelli et al., 2017b; Rodriguez-Manzano et al., 2014; Diez-Valcarce et al., 2012). Both enteric and non-enteric adenovirus types are widely detected in wastewater, in surface water, and in recreational waters, as well as in treated and disinfected waters (Mena and Gerba, 2009). Furthermore, adenoviruses are considered emerging pathogens due to their severe morbidity and the emergence of new variants, and for this reason, they have been included in the drinking water Contamination Candidate List (CCL4) by USEPA (Iaconelli et al., 2017b).

In Italy, no national surveillance programmes exist for HAdVs, and data on their circulation and distribution are scarce since few clinical and environmental studies have been published (La Rosa et al., 2006, 2011; Iaconelli et al., 2017b,a; Wyn-Jones et al., 2011). Unlike other studies, the presence of HAdV was detected only in one river sample at a very low concentration ($<$ LOD) and in 29% of wastewater; higher frequencies were detected in wastewater, in surface water, and in recreational waters, as well as in treated and disinfected water in Spain, Northern Italy, France and the UK (Calgua et al., 2013a,b; Iaconelli et al., 2017a,b; Prevost et al., 2015; Farkas et al., 2018). However, these data are in line with the scarce frequency of this virus found in mussel samples in the same Region (Fusco et al., 2019).

Data on the circulation of SaV in Italy are scarce because this virus has been less investigated than norovirus, the more common virus of the same family (Mancini et al., 2019). Although contaminated waters are a major way of transmission, they have been only recently investigated for the presence of SaV and AsV; in fact, several studies have reported the detection of SaV in wastewater and surface water (Murray et al., 2013; Farkas et al., 2018; Kitajima et al., 2018; Varela et al., 2018) and in mussels (Iritani et al., 2014; Varela et al., 2015; Fusco et al., 2017; Romalde et al., 2018). Furthermore, co-infections of SaVs with multiple enteric viruses (e.g., NoVs, RVs, AsVs, HAdVs, enteroviruses, kobuviruses) have also been reported among cases of acute gastroenteritis (Oka et al., 2015). In our study, SaV prevalence and concentration were higher than that reported for NoVs in all the investigated matrices – groundwater, surface water, sediments and wastewater – suggesting a wide circulation in the environment and a probable underestimation of human cases, compared to prevalence data registered in patients with gastroenteritis (Kitajima et al., 2014). In previous studies, SaV has been identified and characterized in Llobregat River in Spain (Sano et al., 2011), in two South African rivers with higher frequencies (Murray et al., 2013), in surface water from the UK (Farkas et al., 2018) and in sewage samples collected from WWTP in Naples and Palermo in 2011 (Di Bartolo et al., 2013), thus reflecting the extensive circulation of this virus in the population.

Astroviruses have been previously reported in environmental waters (Taylor et al., 2001; Prevost et al., 2015) and have been associated with gastroenteritis in children, in the elderly and in immunocompromised individuals worldwide (Thongprachum et al., 2016). In our study, AsV was the most frequently detected virus in wastewater, reaching an average concentration of 10^4 gc l^{-1} in wastewater effluents. Moreover, unlike what was reported by Farkas et al. (2018), SaV and AsV were detected in river water and sediments with a prevalence similar to those found in mussel samples in the same Region (Fusco et al., 2017). The AsV strains detected and sequenced in our study highlight the presence of human mamastrovirus genotype 1, which shares 98% nucleotide identity with other human AstV strains. Among other viruses, sapovirus and astrovirus could be responsible for several mild episodes of gastroenteritis not requiring hospital care, so their involvement in human gastroenteritis cases could, therefore, be underestimated (Fusco et al., 2017; Mancini et al., 2019).

Aichivirus is considered an emerging pathogen associated with environmental viral contamination and responsible for waterborne and foodborne infections (Kitajima and Gerba, 2015). In our study, AiV was found at variable concentrations in 6.9% of environmental samples (water and sediments) and in 39% of wastewater. Its frequency in river waters is lower than that found in the Sein River and its tributaries (Prevost et al., 2015), although few studies are available in the literature to enable comparison of AiV occurrence in the different environmental matrices and different countries. Sequencing analyses for AiV show that the detected strain belongs to the genotype A with a high correlation (95% nt.id) with some Aichi-1 virus genotype A detected in France in human stools from subjects affected by gastroenteritis (Ambert-Balay et al., 2008). Previous studies described the detection of the Aichi virus 1 genotype A in both sewages and human cases (Lodder et al., 2013; Bergallo et al., 2017) and in mussels in Southern Italy (Fusco et al., 2017), confirming the circulation of this virus in Italy where human cases are rarely reported.

Interestingly, our study highlighted the first report, to our knowledge, of AiV and SaV in environmental samples (water and sediments) in Italy. The detected prevalence suggests a wide spread of these viruses in the environment, probably due to shedding from asymptomatic individuals and/or their involvement in human gastroenteritis cases. However, few data are available in the literature on AiV circulation in the environment and in human gastroenteritis cases.

5. Conclusions

In this study, we highlighted:

- the presence of pathogenic viruses, such as HAV, in river water, which can represent a risk for human health when used for recreational purpose or for irrigation of food and feed;

- among the investigated viruses, RV was the most prevalent and concentrated virus in environmental samples (water and sediments), with AsV being prevalent and concentrated in wastewater. Therefore, as previously reported for this area, HAdV is not feasible for use as an indicator of faecal viral pollution.

This study reports the first detection of SaV and the emergence of AiV in environmental samples (water and sediments) in Italy, indicating the significant circulation of these viruses in the population.

The results of our study confirm the importance of environmental surveillance to evaluate the circulation of pathogens in the environment and the importance of adopting appropriate treatments for raw sewage and drinking water as the only means for the control and prevention of infection deriving from viral contamination of water.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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