ENTERIC VIRUSES, SOMATIC COLIPHAGES AND *VIBRIO* SPECIES IN MARINE BATHING AND NON-BATHING WATERS IN ITALY

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ABSTRACT

Microbial safety of recreational waters is a significant public health issue. In this study we assessed the occurrence and quantity of enteric viruses in bathing and non-bathing waters in Italy, in parallel with microbial faecal indicators, somatic coliphages and *Vibrio* spp.

Enteric viruses (aichivirus, norovirus and enterovirus) were detected in 55% of bathing water samples, including samples with bacterial indicator concentrations compliant with the European bathing water Directive. Aichivirus was the most frequent and abundant virus. Adenovirus was detected only in non-bathing waters. Somatic coliphages were identified in 50% bathing water samples, 80% of which showed simultaneous presence of viruses.

Vibrio species were ubiquitous, with 9 species identified, including potential pathogens (*V. cholerae*, *V. parahaemoylticus* and *V. vulnificus*).

This is the first study showing the occurrence and high concentration of Aichivirus in bathing waters and provides original information, useful in view of a future revision of the European Directive.

Keywords: bathing water; estuary; enteric viruses; somatic coliphages; microbial indicators; *Vibrio* spp

Introduction

Faecal contamination of marine water can lead to potential public health risks for humans due to the presence of enteric pathogens. Waterborne diseases, including gastroenteritis, infections of the upper respiratory tract, and of eyes, ears, and skin, can be acquired by swimmers exposed to contaminated recreational waters.

The global burden of disease associated with bathing in coastal polluted waters has been estimated to be about 120 million cases of gastrointestinal disease and 50 million cases of respiratory diseases each year (Shuval, 2003). More recently, DeFlorio-Barker and coworkers estimated that 90 million illnesses due to recreation events involving surface water occur annually in the United States, resulting in a cost of $2.2- $3.7 billion per year (DeFlorio-Barker et al., 2018). According to a recent systematic review and meta-analysis there is evidence that a number of infections are acquired from bathing in coastal waters, and that bathers have a greater risk of experiencing a variety of illnesses compared with non-bathers (Leonard et al., 2018).

Among waterborne pathogens, viruses are recognized as a major cause of disease outbreaks in recreational waters (Begier et al., 2008; Schets et al., 2018; Sinclair et al., 2009; Yoder et al., 2008a; Yoder et al., 2004; Zlot et al., 2015).

Bathing water quality monitoring was first defined in 1975 in the EU Bathing Water Directive 76/160/EEC (EU, 1976), modified in 2006 by the EU Directive 2006/7/EC (EU, 2006). According to this Directive, fecal indicator organisms (*E. coli*, EC, and Intestinal Enterococci, IE) are used for bathing waters classification. Based on their concentrations, bathing waters are classified as excellent (EC: ≤250 colony forming units (CFU)/100 ml; IE: ≤100 CFU/100 ml, 95th percentile), good EC (≤500 CFU/100 ml; IE: ≤200 CFU/100 ml, 95th percentile), sufficient (EC: ≤500 CFU/100 ml; IE: ≤185 CFU/100 ml, 90th percentile) or poor (values worse than for the latter).

Epidemiological studies support the evidence that enteric and respiratory diseases among bathers increase steadily with increasing concentrations of indicator microorganisms of faecal pollution,

compared with unexposed non-bathers (Shuval, 2003). On the other hand, it is known that indicator microorganisms do not necessarily correlate well with viral pathogens and protozoa, particularly when faecal indicator concentrations are low, since these groups show different survival capacity and transport behaviour (Figueras et al., 1997; Figueras and Borrego, 2010; Ortega et al., 2009).

Enteric viruses are responsible for waterborne disease worldwide, a growing number of them being associated with recreational activities, reviewed in (Bonadonna and La Rosa, 2019; Sinclair et al., 2009). Human viruses most commonly associated with recreational waterborne illnesses are noroviruses (NoV), adenoviruses (AdV), and enteroviruses (EV) (La Rosa et al., 2012).

Adenoviruses, members of the Adenoviridae family, are double-stranded DNA viruses with a worldwide distribution. They are associated with a range of diseases, including respiratory tract syndromes, gastrointestinal, ophthalmologic, genitourinary, and neurologic diseases, reviewed in (La Rosa G. and Suffredini, 2018). In patients with impaired immune responses, the disease can be associated with high morbidity and mortality. Adenoviruses have been detected in various water environments worldwide including wastewater, drinking water, ocean, river and swimming pools (Allard and Vantarakis, 2017). AdV have been found to be responsible for several recreational outbreaks of waterborne illness, the majority of these associated with swimming pools (Sinclair et al., 2009), and have been suggested as possible index organisms for viral pathogens (Gerba et al., 2002; Katayama et al., 2008; Verani et al., 2019). In comparison to RNA viruses, AdVs are more resistant to environmental inactivation and show resistance to many chemical/physical agents and to UV light.

Enteroviruses, members of the *Picornaviridae* family, are classified into four species (EV-A, -B, -C and –D), including both poliovirus and non-polio enteroviruses. They are responsible for a wide spectrum of diseases in people of all ages, including subclinical or mild illness, like colds and fever, or more severe disorders like paralysis, meningitis, and cardiomyopathy. Enterovirus infections are a significant cause of morbidity and mortality throughout the world, in particular for infants and young children; they cause an estimated 10–15 million symptomatic infections per year in the US

alone. Enteroviruses have been detected in different water environments including raw and treated sewage, groundwater, seawater, and fresh water environments (Betancourt and Shulman, 2019; Rajtar et al., 2008). Coxsackievirus and echovirus were found to be responsible for outbreaks associated to viral contamination of lakes, seawater, and swimming pools (Bonadonna and La Rosa, 2019; Sinclair et al., 2009).

Noroviruses are single-stranded RNA viruses belonging to the *Caliciviridae* family, responsible for sporadic and epidemic gastroenteritis in humans. The World Health Organization estimated that norovirus causes annually 685 million cases of acute gastroenteritis, making it the most common cause of acute gastroenteritis worldwide. These viruses are transmitted through the fecal-oral route, leading to high viral loads in sewages. The presence of NoV in sewage and water environments impacted by sewage, including surface waters and seawater, has been widely reported worldwide. Waterborne outbreaks associated with recreational activity in natural waters (lakes, rivers, hot spring) and in pools and fountain have been described (Bonadonna and La Rosa, 2019; Sinclair et al., 2009).

More recently, the occurrence of Aichivirus (AiV) in water environments has raised interest. AiV is a member of the Kobuvirus genus (*Picornaviridae* family), responsible of gastroenteritis in humans through contaminated food or water (Kitajima and Gerba, 2015a). It has been detected in various types of water environments (sewage, river water, and groundwater) worldwide, frequently in higher frequency and greater abundance than other enteric viruses (Haramoto et al., 2018; Kitajima and Gerba, 2015b). However, AiV presence has never been investigated in marine waters.

Somatic coliphages are viruses that infect *E. coli*. The high concentration of coliphages in sewage and other faecal contaminated waters, the easy, fast and economical methods of detection and enumeration, their persistence in water and their resistance to treatments comparable to that of viruses make coliphages good indicators for a wide range of applications. Thus coliphages may also be helpful in the quality control of surface water used for bathing. Possible correlations between the

concentration of coliphages and human viruses in water have been studied, though with disparate results (Gomila et al., 2008; Lucena et al., 2003).

In addition to pathogens from allochthonous sources, also autochthonous marine bacteria can cause disease in humans. Vibrios are natural inhabitants of marine coastal waters and, occasionally, of brackish inland lakes and streams, where they exist as free-living microorganisms and in association to zooplankton and higher organisms (Bonadonna et al., 2002; Esteves et al., 2015; Kirchberger et al., 2016; Neogi et al., 2018; Takemura et al., 2014). Species that cause illness in man include *V. cholerae*, causing gastrointestinal illness (including cholera, generally associated with contaminated seafood), *V. parahaemolyticus*, causing gastroenteritis, *V. vulnificus*, responsible of skin infections, septicaemia and death, and *V. alginolyticus*, etiological agent of wound, ear and eye infections. Wound and ear infections were the most recurrent infections associated with *Vibrio* in bathing water in Europe (Andersson and Ekdahl, 2006; Frank et al., 2006; Schets et al., 2006) and USA (Dziuban et al., 2006; Yoder et al., 2008b) while *Vibrio*-associated gastroenteritis through recreational water exposure is rare (Yoder et al., 2008b).

There is evidence that human *Vibrio* illnesses are increasing worldwide, associated with climatic anomalies, such as heat wave conditions (Baker-Austin et al., 2016; Baker-Austin et al., 2018). The European Centre for Disease Prevention and Control (ECDC) developed a platform to monitor the environmental suitability of coastal waters for *Vibrio* spp. using remotely sensed Sea Surface Temperature (SST) and salinity as an early warning system. In fact, the risk of further *Vibrio* infections is increasing due to climate change (Semenza et al., 2017). The lack of a quantitative correlation between autochthonous species such as vibrios and bacterial indicator parameters suggests that bathing water legislation based on sole faecal indicators does not provide sufficient protection for bathers against vibrios infections (Badley et al., 1990; Dumontet et al., 2000). This fact, combined with the scarcity of recent prevalence data on *Vibrio* species along Italian coasts, support the investigations on the occurrence of these bacteria and the factors affecting their spreading.

Herein we report the results of a study on virological and bacteriological quality of bathing and non-bathing waters in Italy. Occurrence and number of adenovirus, enterovirus norovirus, aichivirus and somatic coliphages were examined in parallel with the traditional faecal bacterial indicators. Contemporaneously, number and types of potentially human pathogenic *Vibrio* species and their relation to environmental conditions (e.g. water temperature and salinity) were investigated.

Material and methods

Study area and sample collection

Two sites, site A on the Adriatic Sea and site B on the Tyrrhenian Sea respectively, were monitored from May to September 2018 (Figure 1). The sampling points were along sandy coasts with shallow sea near the shore. For each site, three sampling points were selected: one in non-bathing waters (point zero) at the estuary of a stream receiving treated urban wastewaters from a Wastewater Treatment Plant (WTP) located less than 1000 meters upstream, and two (points 1 and 2) in the contiguous bathing waters (100-250 mt from the estuary, at its left and at its right). In particular, in site A, the Marano estuary receives the outfall of Riccione WTP and Rimini Nord along-shore area; in site B, the Fosso Grande receives the outfall of Ardea WTP, and is known as polluted channel.

A total of 20 bathing and 10 non-bathing water samples were collected with monthly sampling. Water samples for bacteriological analyses (1 L) and for virological analyses (20 L) were collected in sterile bottles. All samples were taken at a depth of 20 cm below the surface. Environmental conditions (water temperature, salinity and pH) were recorded at sampling. Records of weather and marine conditions were also kept. Samples were transported within the day, under temperature controlled conditions (≤22°C), to the laboratory. Upon arrival, samples were immediately analysed for bacteriological analysis and were stored under refrigerated conditions for virological analyses, that were initiated within 24 hours.

Bacteriological analyses

Detection of *E. coli* and intestinal Enterococci was carried out by miniaturized cultural MPN methods (Colilert 18 and Enterolert E, Idexx, USA).

For *Vibrio* detection, water samples (from 0.1 to 100 mL volumes) were analysed by membrane filtration technique. Membranes were placed on Chromatic™ Vibrio medium (Liofilchem, Italy) and incubated at 37°C for 24-48 hours. Presumptive *Vibrio* colonies were counted separately according to their morphology (mauve colonies for *V. parahaemolyticus*, green blue, turquoise or blue for *V.vulnificus/V.cholerae*, and colourless colonies for *V. alginolyticus*), while colonies with other morphologies (orange or deep violet) were counted but not furtherly tested. Details of the selection and screening procedure are reported in Supplementary Material. Depending on the abundance of the specific colony type, a percentage ranging from 10 to 25% of the colonies of the same morphology was isolated from each plate and subjected to biochemical confirmation tests (Gram staining, oxidase test, and string test). Some biochemical positive isolates (about 40%) were identified by miniaturized kits (bioMerieux, France) while the others were subjected to molecular identification. Briefly, for each isolate, a single colony was suspended in ultrapure water (0.2 mL), heat-lysed (95 °C for 10 min), centrifuged to sediment debris (2 min at 10.000 × *g*) and 1.5 µL of the supernatant was used for the PCR amplification of the *rpoB* gene zhang (Ki et al., 2009). The 730 bp PCR products were purified with Montage PCRm96 Micro well Filter Plates (Millipore, Burlington, USA) and DNA sequencing was performed on both strands (BioFab Research, Rome). Consensus sequences were assembled from the raw forward and reverse electropherograms, using the Molecular Evolutionary Genetics Analysis (MEGA) software, version 7.0. Species identification was evaluated using NCBI BLAST (Basic Local Alignment Search Tool, available at: http://blast.ncbi.nlm.nih.gov/Blast.cgi).

In addition to *Vibrio* spp. count, a species-specific enumeration of *V. parahaemolyticus* was performed by colony hybridization. Briefly, 10 ml of each sample were high-speed centrifuged (≥10.000 × *g* for 30 min) to sediment seawater bacteria, the upper 9 ml of water were carefully withdrawn and the remaining milliliter was vortexed to resuspend bacteria, spread on trypticase soy

agar medium added with NaCl to 3% (TSA-S), and subsequently incubated at 37°C for 18 h. *V. parahaemolyticus* enumeration was then performed as previously described (Suffredini et al., 2014).

Virological analyses

Somatic coliphages were detected by single-agar-layer (SAL) plaque assay, according to the USEPA Method 1602. *E. coli* ATCC 13706 strain was used in the assay. Five mL aliquots of each sample were directly analysed. After an overnight incubation, plaques were counted and summed for all plates from a single sample. The quantity of coliphages in a sample was expressed as plaque forming units (PFU)/100 mL.

Enteric viruses were concentrated using the adsorption-elution procedure with electronegative filters (La Rosa et al., 2017a). Before concentration, all samples were artificially contaminated with Mengovirus used as a sample process control. The recovery rate for Mengovirus was calculated as the ratio between the number of genome copies (g.c.) that were recovered after concentration and the g.c. of viral stock used to spike the samples, as previously described (Costafreda et al., 2006). Before concentration, the pH of the sample was adjusted at 3.5 with H_2SO_4; then, 20 L of water were flowed through a standard filter apparatus containing a sterile electronegative filter (Sartorius Membrane Filter Cellulose Nitrate 11306-142-G), using a peristaltic pump and a flow rate of 0.5 L/min. For virus elution, 50 mL of 3% beef extract pH 9.5 was recirculated through the filters for 20 min. The pH was then neutralized with HCl 1N. A secondary concentration step was performed by PEG precipitation. PEG 6000 and NaCl were added to reach final concentrations of 10% and 1.6% w/v, respectively. The mix was incubated at 4°C for 14–18 h and then centrifuged at 7000 × *g* for 30 min at 4°C. The supernatant was discarded, and the pellet dissolved in 10 mL PBS pH 7.4. The concentrate was divided into two aliquots of 5 mL, one of which immediately subjected to genome extraction, the other stored at -80 °C for viral cell culture purposes.

Nucleic acid extraction and purification were performed using the NucliSens extraction kit (BioMerieux, Paris, France) according to the manufacturer's instructions, and eluted nucleic acids

(100 µL) were stored at − 80°C until molecular analysis. Following RNA/DNA extraction, all samples underwent PCR amplification by real-time RT-qPCR to detect and quantify NoV, EV, AiV, and by real-time qPCR for AdV. Analysis for NoV, EV, AiV and AdV were conducted with previously described reaction conditions (Fuhrman et al., 2005; Hernroth et al., 2002; ISO, 2017; Kitajima et al., 2013) and the panel of primers and probes reported in Table 1. Each sample was assayed in duplicate using 5 µL of undiluted nucleic acid extract in a final volume of 25 µL. Two negative controls were included in each assay. Presence of PCR inhibitors was ruled out using an external amplification control (in vitro synthesized RNA) as described in ISO 15216 (ISO, 2017). Samples showing an inhibition ≥ 50% were subjected to further purification by mean of repetition of the DNA/RNA binding and washing steps of the NucliSens extraction system. Tenfold dilutions of plasmids DNA containing the region targeted by real-time PCR primers were used for standard curve construction (acceptability criterion: slope between -3.1 and -3.6 and R^2 correlation coefficient ≥0.98). All RT-qPCR reactions were conducted using the UltraSense one-step qRT-PCR System (Invitrogen), while qPCR reactions were prepared using the Hydra Probe qPCR Master Mix (BioLab, Italy). Molecular biology water served as a non-template control. Real-time PCR were conducted on a QuantStudio 12K instrument (Thermo Scientific).

Qualitative nested (RT)-PCR assays were performed for molecular characterization of enteric viruses. NoV (GI and GII), EV, AdV, and AiV were analysed using broad range primers shown in Table 1. A final mixture of 25 µL was prepared for the first PCR with 2 µL of RNA and 10 pmol of forward and reverse primers, using the MyTaq One Step RT-PCR kit (Bioline Ltd, London, UK) for EV detection and the SuperScript™ IV One-Step RT-PCR System (Invitrogen) for NoV GI, NoV GII and AiV detection. The nested PCR was performed in 25 µL of PCR reactions with the MyTaq Red Mix (Bioline Ltd, London, UK) and Platinum Green Hot Start PCR Master Mix (Invitrogen) respectively, using one µl of the product from the first cycle as a template. For AdV, the first and the nested cycles were both prepared using the MyTaq Red Mix. Standard precautions were taken to prevent cross-contamination of samples. Positive and negative controls were regularly used.

PCR products of the expected size were purified with Montage PCRm96 Micro well Filter Plates (Millipore, Burlington, MA, US) and confirmed by sequencing both strands (BioFab Research). Consensus sequences were assembled as previously described using the MEGA software v7.0. The relatedness of sequences was evaluated using NCBI BLAST (Basic Local Alignment Search Tool, available at: http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Cell culture for enteric viruses

Cell cultures were used to assess the infectivity of adenovirus using A549 cells. To this end, 5 mL of PEG/concentrated water samples were decontaminated with chloroform and added to 7 mL of the culture medium, which was added to A549 cells (ECACC) cultured in Dulbecco's modified Eagle's medium (Euroclone) supplemented with 2% of fetal bovine serum (Euroclone). The cell cultures were incubated at 37°C with 5% of CO_2 and observed daily under an optical microscope for 2 weeks until detection of cytopathic effects, followed by two subsequent confirmation steps (Carducci et al., 2009).

Statistical analysis

Descriptive statistics (average, median, standard deviation, etc.) were calculated for environmental parameters, bacteriological parameters (fecal indicators, *Vibrio* spp.) and somatic coliphages using the XlStat software (v 2019.1.1, Addinsoft, Boston, US). To assess the degree of agreement (DoA) between bacteriological and virological results, inter-rater reliability (expressed as Kappa) was calculated comparing detection of enteric viruses to: i) detection of coliphages; ii) detection of fecal indicators at levels corresponding to a bathing water class 'poor' according to the EU Directive; iii) detection of fecal indicators at levels corresponding to a bathing water class equal or worse than 'sufficient' according to the EU Directive. Based on Kappa results, the DoA was classified as slight (0–0.20), fair (0.21–0.40), moderate (0.41–0.60), substantial (0.61–0.80) or almost perfect (0.81–1.00).

Moreover, correlation of quantitative values of *E. coli*, intestinal Enterococci, somatic coliphages and enteric viruses in bathing waters was assessed through calculation of the Pearson correlation coefficient (*r*). For this calculation AiV were selected among enteric viruses, being the group with the highest number of positive, quantifiable results. Pearson correlation of quantitative values of *Vibrio* spp., *E. coli*, intestinal Enterococci and physical-chemical parameters in bathing waters was also performed.

Finally, to assess the accuracy of bacteriological parameters in predicting the presence of enteric viruses in bathing waters, the positive predictive value (PPV) and the negative predictive value (NPV) were calculated for the aforementioned three categories (coliphages, fecal indicators class 'poor', fecal indicators class 'sufficient' or worse). Kappa values, PPV and NPV were calculated using the GraphPad Software QuickCalcs online tool (https://www.graphpad.com/).

Results

Environmental parameters

Environmental conditions at sampling time are reported in Supplementary Material. In bathing sites, water temperature, pH and salinity were, on average, 23.7 °C (range 19.9 °C – 28.8 °C), 8.23 (range 8.00 – 8.68) and 33.8‰ (range 26.0‰ – 40.0‰), respectively, with no significant difference between the two sampling sites on the Adriatic Sea and the Tyrrhenian Sea. In estuaries, both average water temperature and pH were close to those registered in the adjacent bathing areas (23.1 °C and 8.01, respectively) while, as presumable, salinity was significantly lower (20.2‰).

Bathing waters

The level of microbial indicators in bathing waters met the requirements established by the EU Directive in 14 out of 20 samples (70%), while 6 samples (4 from the Tyrrhenian and 2 from the Adriatic coast), collected in July (*n*=1), August (*n*=4), and September (*n*=1) exceeded the limits set

for ‘sufficient’ waters for *E. coli* and/or Enterococci, and were therefore of poor quality (Table 2 and Figure 2).

Quantitative and qualitative results for enteric viruses and somatic coliphages are summarized in Table 2. For virological analysis, sample recovery ranged from 0.7% to 95.6%, with an average of 14.8%. PCR inhibition was present in 6 out of 30 samples, which required an additional RNA purification to remove inhibitors. Combining results obtained by nested and real-time PCR, enteric viruses were detected in 11/20 (55%) bathing water samples, of which 6 compliant with the Directive for microbial indicators (status of at least ‘sufficient’) and therefore suitable for bathing according to the current legislation. Norovirus was detected in 5/20 (25%) bathing water samples, with four GII-positive and one GI-positive samples, the latter characterized by sequencing as GI.4. Viral loads for NoV were overall low, the highest concentration being 3.2 genome copies (g.c.)/L. Aichivirus was detected in 6/20 (30%) bathing water samples with high viral loads, compared to noroviruses, reaching up to 1100 g.c./L. All AiV-positive samples were characterized as AiV-1-B by amplicon sequencing. One sample was positive for EV (5%), but genome copies were below the quantification limit; AdV was never detected in bathing waters.

Somatic coliphages were detected in 10/20 bathing water samples (Table 2), with higher counts found in August (up to 2120 PFU/100 mL) and an average of 36 PFU/100 mL and 280 PFU/100 mL in the Adriatic and Tyrrhenian coasts, respectively.

Simultaneous presence of viruses and coliphages was detected in eight bathing water samples. Overall, the degree of agreement (DoA) between these two parameters was moderate (Kappa = 0.500) and comparable to the DoA of enteric viruses and classification of bathing areas (waters classification ‘poor’: Kappa= 0.327, DoA fair; waters classification equal or worse than ‘sufficient’: Kappa= 0.510, DoA moderate). Similarly, a predictive value for the presence of enteric viruses of 80.0% and 87.5% (PPV) and 70.0% and 66.7% (NPV) was shown by somatic coliphages and by a class equal or worse than ‘sufficient’ of bathing waters, respectively. Given the limited extension of sampling in the present study, a larger number and variety of samples from bathing waters should

be tested to assess predictive capacity of these factors with regard to presence of enteric viruses. Quantitative levels of enteric viruses (AiV) did not show any significant correlation with other microbial parameters in bathing waters (correlation coefficient *r*= -0.028, -0.059 and -0.048 with EC, IE and somatic coliphages, respectively). On the other hand, a weak and a strong correlation was found between somatic coliphages and EC (*r*= 0.162) or IE (*r*= 0.934), respectively. No correlation was also found among bacterial indicators concentration and environmental parameters as water temperature, salinity and pH (correlation coefficient *r* ranging between -0.271 and 0.023; see Supplementary Material).

Vibrio species were ubiquitous and numerous in bathing sites, where they were detected in 17/20 samples (85%), with concentrations ranging from undetectable to 1.7×10^4 CFU/100 mL (Table 3). Higher counts were detected from July to September and particularly in August (Figure 3, panel A), when concentrations of 10^4 CFU/100 mL were exceeded in the seashore of Riccione. Overall, the mean concentration of *Vibrio* spp. in the Adriatic Sea was of the same magnitude order than along Tyrrhenian coast (4.2×10^3 and 1.3×10^3 CFU/100 mL, respectively). No significant correlation was found among *Vibrio* spp. concentration and fluctuations of temperature and salinity (correlation coefficient *r*= -0.192 and 0.069 with temperature and salinity, respectively), while a positive correlation was found between *Vibrio* spp. and *E. coli* in bathing areas (correlation coefficient *r*= 0.861, see Supplementary Material).

A total of eight species were identified by sequencing analysis in bathing waters: *V. alginolyticus*, *V. campbellii*, *V. cholerae*, *V. diabolicus*, *V. harveyi*, *V. owensii/hyugaensis, V. parahaemolyticus,* and *V. rotiferanius*. The most frequently detected species was *V. harvey* (13/20 samples), followed by *V. cholerae* and *V. parahaemolyticus*, equally detected (9 samples). With regard to the latter, species-specific counts showed low concentrations, ranging from undetectable to 20 CFU/100 mL, with the highest values corresponding to samples taken in August in the Ardea site (data not shown).

Non-bathing waters

Microbial indicators in the samples collected at the estuaries of the streams were, as expected, higher than in bathing waters (Table 2 and Figure 2), exceeding 4800 MPN/100 mL for EC in August, and reaching 2827 MPN/100 mL for IC in September. However, values below the detection limit for both EC and IC were recorded for some samples in May and in June.

Enteric viruses were detected in 7/10 (70%) non-bathing waters. NoV was detected in 3/10 (30%) samples, with low viral loads (up to 13 g.c./L), while Aichivirus was detected in 6/10 (60%) samples with viral loads up to 1860 g.c./L, all characterized as AiV1-B by amplicon sequencing. Adenovirus, characterized as type 41, was detected in two estuarine samples (20%); cell culture of these sample concentrates confirmed the absence of viable or culturable adenoviruses.

Somatic coliphages were detected in 7/10 non-bathing waters (up to 3560 PFU/100 mL in August) and an average of 172 PFU/100 mL and 840 PFU/100 mL at the site of Riccione and at the site of Ardea, respectively. Simultaneous presence of enteric viruses and somatic coliphages was detected in six samples.

Vibrio species were detected abundantly also in estuary samples (8/10 samples), with loads up to 1.0×10^4 CFU/100 mL and an average concentration of 2.7×10^3 CFU/100 mL (Table 3). High counts were obtained in July in the site of Riccione and *Vibrio* concentrations showed an irregular trend during the sampling period (Figure 3, panel B). Correlation of *Vibrio* and *E. coli* counts (r=0.739) was confirmed as per bathing waters. In these samples the species detected included *V. alginolyticus, V. campbellii, V. cholerae, V. harveyi, V. owensii/hyugaensis, V. parahaemolyticus,* and *V. vulnificus*, with the prevalence of the first one.

Discussion

Currently, microbiological quality monitoring of bathing waters is solely based on the measurement of faecal indicator organisms, *Escherichia coli* and intestinal enterococci, as an alternative to pathogens detection. Moreover, the number of viral pathogens potentially responsible for waterborne illness is significant and therefore testing for all potential hazards could not be feasible.

In this study, a survey was conducted to determine enteric viruses' occurrence and quantity in recreational waters together with faecal indicator bacteria, autochthonous bacteria such as vibrios and somatic coliphages. Assessing the presence of viruses in water is not defined by the current legislation. The previous EU Bathing Water Directive 76/160/EEC (EU, 1976) recommended enteroviruses among parameters to be tested, requiring that 95% of samples taken during the bathing season should not contain enteroviruses in 10 L of water. The current Bathing Water Directive 2006/7/EC identifies only two parameters for the monitoring and assessment of bathing water quality and for their classification, intestinal enterococci and *Escherichia coli*.

Results of the present study confirm previous finding showing that microbiological parameters defined in the current Bathing Water Directive have low predictive capability for the presence of human viruses in coastal waters. Indeed, enteric viruses were found in 11/20 (55%) of bathing water samples, including samples considered safe based on microbial indicator concentrations. Interestingly, AiV were the most frequent and numerous virus detected along both the sampling sites, found in 30% of bathing water samples and 60% of non-bathing water samples with high viral loads (up to 1100 g.c./L and 1860 g.c./L, respectively). To our knowledge, AiV presence in bathing waters has never been demonstrated before. These viruses have been detected, however, in various types of environmental samples worldwide, such as sewage, river water, groundwater, and shellfish, in higher frequency and greater abundance than other human enteric viruses, suggesting that it could potentially be proposed as an indicator of viral contamination in the environment (Kitajima and Gerba, 2015b). In Italy, we recently found AiV in 98% of urban wastewater samples with concentrations reaching 2.3×10^6 genome copies/L (Suffredini et al., 2019), suggesting that AiV can likely contaminate other water environments impacted by sewage, including bathing waters. Moreover, AiV were detected in 8% of mussels collected in harvesting areas in the Campania region in Southern Italy (Fusco et al., 2017), suggesting a diffuse presence of these viruses in marine waters. The genotypes distribution detected in environmental water samples varies in different geographical regions. In this study only AiV1-B was detected, which is the genotype

usually more commonly detected in the European countries (Di Martino et al., 2013; Lodder et al., 2013) and South America (Alcala et al., 2010; Burutaran et al., 2016) while genotype A is more frequently detected in Asia (Kitajima et al., 2011; Kitajima and Gerba, 2015b) and Africa (Sdiri-Loulizi et al., 2010).

Adenovirus was detected in two samples in non-bathing areas but was never detected in bathing water samples. Among the waterborne viruses, AdV can be considered as an index pathogen, owing to its abundance in sewage and persistence in the environment (Verani et al., 2019). During the EU FP6 Project VIROBATHE (2005-2007), AdV appeared to be a promising viral indicator for bathing water quality, since it was detected in 36% of recreational (fresh and marine) waters in the participant European countries (Wyer et al., 2012; Wyn-Jones et al., 2011), with loads of 3.2×10^3 g.c./L water on average (and up to 9.1×10^4) in marine samples. Moreover, 47% of marine waters positive for AdV contained infectious viruses. More recently, Verani and co-workers found AdV in 21% of seawater samples in Italy, with concentration up to 10^2 g.c./L (Verani et al., 2019). In the light of these results, the absence of positive samples in bathing waters in this study was unexpected. To confirm negative results, different molecular tests were used (date not shown), and inhibition in negative samples was excluded. It is however important to consider that negative findings might also be the result of the small sample size of the study.

Norovirus was detected in 25% of bathing water samples, at low concentrations, also in samples considered safe based on microbial indicators concentrations. The VIROBATHE Project detected NoV in 9.4% of recreational water samples, and almost all the norovirus GI-positive samples were found in four sites in Italy (Wyn-Jones et al., 2011). More recently, NoV was detected in seawater samples from shellfish production areas and water samples from nearby underwater sewage discharge points in Italy (La Rosa et al., 2017b). Our study therefore confirms previous studies showing NoV circulation in seawater.

Enteroviruses were detected only in one bathing water sample. In Italy, EV have been identified in raw and treated wastewaters (La Rosa G. et al., 2010; Pellegrinelli et al., 2013; Pellegrinelli et al.,

2017; Pennino et al., 2018), river (La Rosa et al., 2017c), groundwater used for irrigation (De Giglio et al., 2017). Only one study shows the presence of enteroviruses in seawater samples in Italy. Positive samples were detected in 32.6% by cell culture techniques, most frequently in the summer months (Pianetti et al., 2000).

In the present study, 30% of bathing water samples positive for enteric viruses showed safe counts for *E. coli* and Enterococci, therefore suitable for bathing according to the current legislation. The presence of enteric viruses in recreational waters compliant for bacterial indicators is reported worldwide (Love et al., 2014; Wyn-Jones et al., 2011), indicating that current standards defined in the Bathing Water Directive, based on EC/IE levels, have low predictive capability for the presence of human viruses in these waters. Testing for somatic coliphages presents an alternative to testing for viral pathogens, as they share similar morphologies, are more environmentally stable than faecal indicator microorganisms, are numerous in human faecal waste, and can be detected by simple and inexpensive culture-based detection methods. Different studies suggest a likely relationship between coliphages and increased risk of gastroenteritis for bathers (Abdelzaher et al., 2011; Griffith et al., 2016; Wiedenmann et al., 2006). However, reliable data on the meaning of enumerating coliphages to predict the presence of intestinal pathogens, in particular viruses, are contradictory and often incomplete. In this study a total of 14 samples (47%) showed simultaneous presence of somatic coliphages and enteric viruses, and coliphages detection showed a positive predictive value for enteric viruses presence of 80.0%, though similar predictive values were obtained with bacterial fecal indicators when using more strict contamination thresholds. Given the limitation of sampling size in this study, a predictive capacity of coliphages towards virus presence in bathing waters should be assessed through more extensive surveys or through meta-analysis studies, so as to include a wider variability of bathing sites and environmental conditions.

Because of their ubiquity and their potential as pathogens *Vibrio* were also investigated in this study. These bacteria are autochthonous in marine and estuarine environments (Baker-Austin et al., 2018; Suffredini and Caburlotto, 2015). They are also commonly present in shellfish and other

seafood (Froelich and Noble, 2016; Huehn et al., 2014; Odeyemi, 2016; Romalde et al., 2014). The *Vibrio* genus includes more than 100 species (Romalde et al., 2014), present in the environment as free-living or associated with different substrata. Some species are associated with human cases of ear and wound infections, gastroenteritis, caused by ingestion of seafood or contact with *Vibrio* containing water. The most significant pathogenic *Vibrio* is *V. cholerae*, the aetiological agent of epidemic cholera, *V. parahaemolyticus,* responsible for seafood-associated gastroenteritis worldwide, and *V. vulnificus*, microorganism that may cause septicaemia and serious wound infections in susceptible individuals. All these tree pathogenic *Vibrio* species were detected in this study, confirming the wide distribution of these microorganisms in estuarine and coastal waters of Italy (Caburlotto et al., 2012; Gugliandolo et al., 2005; Masini et al., 2007; Ottaviani et al., 2013). There is substantial evidence that *Vibrio*-associated diseases are increasing worldwide, partially owing to increased geographic distribution of the pathogenic species favoured by climate changes and rising seawater temperatures (Baker-Austin et al., 2017; Baker-Austin et al., 2018; Vezzulli et al., 2016).

In this study *Vibrio* counts ranging from undetectable to 1.7×10^4 CFU/100 mL and an average concentrations of 4.7×10^3 CFU/100 mL were found in bathing waters. Concentrations up to 10^5 and 10^6 CFU/100 mL were previously reported in waters from the Tyrrhenian and Adriatic Sea (Bonadonna et al., 2002; Masini et al., 2007). Similarly, quantitative levels ranging ranging from undetectable to 2.4×10^4 CFU/100 mL or to 1.9×10^6 CFU/100 mL were described in recreational waters in The Netherland and in Germany, respectively (Boer et al., 2013; Schets et al., 2011). The presence and the quantitative levels of *Vibrio* spp. in water bodies depends on multiple environmental factors (Johnson, 2015). Although the effects of environmental parameters are highly species dependent, in general, temperature, salinity and pH are considered the most important. Indeed, in a survey carried out along the Adriatic Sea coasts (Bonadonna et al., 2002), not far from those considered in this study, a correlation between *Vibrio* counts and both temperature and salinity was observed. In this study a quantitative relation between water temperature and *Vibrio*

concentration in bathing water was not observed probably for the low range of temperature recorded during the sampling period (from 19 to 29°C). The same lack of correlation was also observed in the course of a study conducted from May to September in Netherlands (Schets et al., 2006). However, consistent with studies highlighting the seasonality of *Vibrio* presence and levels (Boer et al., 2013; Croci et al., 2001; Mookerjee et al., 2015; Paranjpye et al., 2015; Takemura et al., 2014), this study also recorded an increase in concentrations during the summer months (in August, in particular). No correlation between vibrios concentration and salinity was also observed. Probably in our study the salinity does not vary enough to identify a meaningful relationship. Salinity effects are often very complex, and can include covariance with other parameters, which makes it difficult to fully separate potential relationships (Johnson, 2015). In this study the pH values of bathing water were within a narrow range, from 8 to 8.68. These conditions respected the limits reported by scientific literature for an optimal vibrios growth. In fact, it is known that *Vibrio* grows best under alkaline conditions and most species grow between pH 6.5 and 9.0 (Percival and Williams, 2014). In this investigation vibrios levels were positively associated to faecal indicators concentration; positive correlations were also found in other studies (Bonadonna et al., 2002; Viau et al., 2011). Nevertheless, the natural ubiquity of vibrios in seawaters makes their presence not necessarily related to faecal contamination events, then their relation with faecal indicators is complex and influenced by multiple factors (Bonadonna et al., 2002). Consequently, the adequacy of faecal indicators into represent *Vibrio* species in seawater is at present still object of discussion.

In conclusion, the results of this study provide an overview of the viral contamination of seawaters used for recreational purposes in coastal waters. Detection of viruses in the absence of a high numbers of bacterial indicators confirms the need to carefully assess risks to human health in bathing waters affected by watercourses affected by sewage discharges. On the other hand, the presence of autochthonous vibrios can represent a constant and persistent health risk regardless of the presence of effluents along the coast, especially for immunocompromised people.

Aichivirus was detected for the first time in marine waters, in greater abundance and higher frequency than other enteric viruses. Further studies are needed in order to investigate the potential of AiV to be used as indicator for marine waters.

The future Bathing Water Directive will have to carefully assess all microbial potential risks, also in the light of the ongoing climate changes that will affect not only the marine environmental conditions of the Mediterranean countries but also the Northern regions of Europe.

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Conflicts of interest

There are no conflicts of interest to declare.

Table 1: Primers and probes used in the study for real-time (RT)-qPCR and nested (RT)-PCR of viral targets and for PCR for species identification of *Vibrio* spp.

Target	PCR	Primer/probe name and sequence (5'-3')		Target region	Amplicon (bp)	Reference
NoV GI	real-time RT(q)PCR	QNIF4	CGCTGGATGCGNTTCCAT	ORF1-ORF2 junction	-	(da Silva et al., 2007; Hoehne and Schreier, 2006; Svraka et al., 2007)
		NV1LCR	CCTTAGACGCCATCATCATTTAC			
		TM9	FAM-TGGACAGGAGATCGC-MGB			
NoV GII	real-time RT(q)PCR	QNIF2	ATGTTCAGRTGGATGAGRTTCTCWGA	ORF1-ORF2 junction	-	(Kageyama et al., 2003; Loisy et al., 2005)
		COG2R	TCGACGCCATCTTCATTCACA			
		QNIFs	FAM-AGCACGTGGGAGGGCGATCG-TAMRA			
AdV	real-time (q)PCR	AdV-upstream	CWTACATGCACATCKCSGG	Hexon	-	(Hernroth et al., 2002)
		AdV-downstream	CRCGGGCRAAYTGCACCAG			
		AdV-ACDEF	FAM-CCGGGCTCAGGTACTCCGAGGCGTCCT-TAMRA			
AiV	real-time RT(q)PCR	AiV-AB-F	GTCTCCACHGACACYAAYTGGAC	VP0	-	(Kitajima et al., 2013)
		AiV-AB-R	GTTGTACATRGCAGCCCAGG			
		AiV-AB-TP	FAM-TTYTCCTTYGTGCGTGC-MGB			
EV	real-time RT(q)PCR	EV1	GATTGTCACCATAAGCAGC	5'NTR	-	(Fuhrman et al., 2005)
		EV2	CCCCTGAATGCGGCTAATC			
		EV-probe	FAM-CGGAACCGACTACTTTGGGTGTCCGT-BHQ			
NoV GI	RT nested PCR	COG1F	CGYTGGATGCGNTTYCATGA	ORF2	1st cycle, 381 nested, 318	(Kageyama et al., 2003; Kojima et al., 2002)
		G1-SKR	CCAACCCARCCATTRTACA			
		G1-SKF	CTGCCCGAATTYGTAAATGA			
		G1-SKR	CCAACCCARCCATTRTACA			
NoV GII	RT nested PCR	COG2F	CARGARBCNATGTTYAGRTGGATGAG	ORF2	1st cycle, 387 nested, 344	(Kageyama et al., 2003; Kojima et al., 2002)
		G2-SKR	CCRCCNGCATRHCCRTTRTACAT			
		G2-SKF	CNTGGAGGGCGATCGCAA			
		G2-SKR	CCRCCNGCATRHCCRTTRTACAT			
AdV	nested	AdhexF1	TICTTTGACATICGIGGIGTICTIGA	Hexon	1st cycle,	(Lu and Erdman, 2006)

	PCR	AdhexR1	CTGTCIACIGCCTGRTTCCACA		764-896 bp	
		AdhexF2	GGYCCYAGYTTYAARCCCTAYTC		nested, 688-821 bp	
		AdhexR2	GGTTCTGTCICCCAGAGARTCIAGCA			
AiV	RT nested PCR	AiV 6290	ACACTCCCACCTCCCGCCAGTA	3C/D junction	1st cycle, 312 bp	(Oh et al., 2006); Suffredini et al, 2019)
		AiV 6602	AGGATGGGGTGGATRGGGGCAGAG			
		AiV 6309	GTACAAGGACATGCGGCG		nested, 279 bp	
		AiV R2modified	GGGGCAGAGAATCCRCTC			
EV	RT nested PCR	ET1	CAAGCACTTCTGTTTCCCCGG	5'NTR	1st cycle, 440 bp	(Zoll et al., 1992)
		ET3	ATTGTCACCATAAGCAGCCA			
		ET2	TCCTCCGGCCCCTGAATGCG		nested, 155 bp	
		ET3	ATTGTCACCATAAGCAGCCA			
***Vibrio* spp.**	PCR	qVb-F1731	GTTTGCGCGTTGTAACGAGTAC	*rpoB*	730 bp	(Ki et al., 2009)
		qVb-R2460	CTCAGATACTAAGATCGAGTCTTCG			

FAM: 6-carboxyfluorescein; TAMRA: 6-carboxytetramethylrhodamine; MGB: minor groove binder/non-fluorescent quencher; BHQ: black hole quencher

Table 2: Results of the analysis for indicator microorganisms, enteric viruses and somatic coliphages

Site	Sample ID	Type of water	Sampling date	E. coli (MPN/100 mL)	Enterococchi (MPN/100mL)	Viruses quantification (g.c./L) and characterization					Somatic coliphages (PFU/100mL)
						NoV GI	NoV GII	EV	AiV	AdV	
	Riccione (Adriatic Sea)										
A-0	2667	Estuary	29/05/2018	<1	<1	-	-	-	-	-	-
A-1	2668	Bathing	29/05/2018	<1	<1	-	-	-	-	-	-
A-2	2669	Bathing	29/05/2018	<1	<1	-	-	-	-	-	-
A-0	2683	Estuary	18/06/2018	<1	15	-	-	-	-	-	-
A-1	2684	Bathing	18/06/2018	445	104	-	-	0,4	-	-	20
A-2	2685	Bathing	18/06/2018	<1	10	-	-	-	-	-	-
A-0	2700	Estuary	10/07/2018	2827	387	13,0	0,6	-	-	*AdV-41*	640
A-1	2701	Bathing	10/07/2018	45	10	-	0,3	-	-	-	20
A-2	2702	Bathing	10/07/2018	44	24	-	2,1	-	1100,2 *AiV1-B*	-	-
A-0	2709	Estuary	27/08/2018	1900	190	-		-	964,7	-	220
A-1	**2710**	**Bathing**	**27/08/2018**	**2100**	**230**	**-**	**3,2**	**-**	**-**	**-**	**40**
A-2	**2711**	**Bathing**	**27/08/2018**	**>4800**	**150**	**-**	**-**	**-**	**121,5**	**-**	**260**
A-0	2720	Estuary	26/09/2018	41	163	-	-	-	1860,9 *AiV1-B*	-	-
A-1	2721	Bathing	26/09/2018	34	185	-	-	-	-	-	-
A-2	2722	Bathing	26/09/2018	261	55	-	-	-	111,9 *AiV1-B*	-	1
	Ardea (Tyrrhenian Sea)										
B-0	2664	Estuary	30/05/2018	2406	275	-	-	-	-	-	200
B-1	2665	Bathing	30/05/2018	111	40	-	-	-	-	-	-
B-2	2666	Bathing	30/05/2018	58	52	-	-	-	-	-	-
B-0	2686	Estuary	24/06/2018	17	76	-	-	-	*AiV1-B*	-	20
B-1	2687	Bathing	24/06/2018	4	60	-	-	-	-	-	20
B-2	2688	Bathing	24/06/2018	<1	<1	-	-	-	*AiV1-B*	-	-

B-0	2697	Estuary	04/07/2018	1300	107	0,8	-	-	1203,0 *AiV1-B*	*AdV-41*	180
B-1	2698	Bathing	04/07/2018	8	10	-	-	-	*AiV1-B*	-	-
B-2	**2699**	**Bathing**	**04/07/2018**	**1600**	**150**	**-**	**0,1**	**-**	**-**	**-**	**20**
B-0	2712	Estuary	28/08/2018	>4800	1900	-	-	-	122,7 *AiV1-B*	-	3560
B-1	**2713**	**Bathing**	**28/08/2018**	**270**	**370**	**-**	**-**	**-**	**-**	**-**	**320**
B-2	**2714**	**Bathing**	**28/08/2018**	**870**	**920**	***NoV GI.4***	**-**	**-**	**-**	**-**	**2120**
B-0	2717	Estuary	25/09/2018	1203	2827	-	0,2	-	144,7	-	12
B-1	**2718**	**Bathing**	**25/09/2018**	**354**	**304**	**-**	**-**	**-**	**413,2**	**-**	**16**
B-2	2719	Bathing	25/09/2018	28	49	-	-	-	-	-	-

Samples exceeding the limits of EU legislation for bathing waters are highlighted in bold.

Results of real-time (RT)-qPCR are reported as concentrations of the target virus (c.g./L); results of the nested (RT)-PCR (in italics) are reported as the type/genotype detected. Samples in which the viral target was not detected are reported as "-".

Table 3: Qualitative and quantitative assessment of *Vibrio* spp. in the monitored costal sites

Site	*Vibrio* spp. positive samples (%)	*Vibrio* spp. concentration (CFU/100 mL)				*Vibrio* species detected
		Min	Max	Avg	SD	
Riccione (Adriatic Sea)						
Estuary	3/5 (60)	<1	1.0×10^4	2.9×10^3	4.4×10^3	*V.campbellii, V.cholerae, V.harveyi, V.owensii/V.hyugaensis, V.parahaemolyticus, V.vulnificus*
Bathing	8/10 (80)	<1	1.7×10^4	4.2×10^3	6.4×10^3	*V.alginolyticus, V.campbellii, V.cholerae, V.diabolicus, V.harveyi, V.owensii/V.hyugaensis, V.parahaemolyticus*
Ardea (Tyrrhenian Sea)						
Estuary	5/5 (100)	1.6×10^2	5.8×10^3	2.5×10^3	2.7×10^3	*V.alginolyticus, V.campbellii, V.cholerae, V.harveyi, V.vulnificus*
Bathing	9/10 (90)	<1	4.3×10^3	1.3×10^3	1.4×10^3	*V.alginolyticus, V.campbellii, V.cholerae, V.diabolicus, V.harveyi, V.owensii/V.hyugaensis, V.parahaemolyticus, V.rotiferianius*
Total						
Estuary	8/10 (80)	<1	1.0×10^4	2.7×10^3	3.5×10^3	*V.alginolyticus, V.campbellii, V.cholerae, V.harveyi, V.owensii/V.hyugaensis, V.parahaemolyticus, V.vulnificus*
Bathing	17/20 (85)	<1	1.7×10^4	2.8×10^3	4.7×10^3	*V.alginolyticus, V.campbellii, V.cholerae, V.diabolicus, V.harveyi, V.owensii/V.hyugaensis, V.parahaemolyticus, V.rotiferianius*

Min = minimum value; Max = maximum value; Avg = average; SD = standard deviation

Figure 1: GIS map of the collection sites

Figure 2: *E. coli* and *Enterococci* values of bathing and non-bathing waters

Figure 3: *Vibrio* spp. loads in the bathing waters of the two sampling sites in relation to the environmental parameters.

Panel A: bathing waters, Panel B: non-bathing waters. Vibrio spp. counts and environmental parameters (temperature, salinity and pH) are reported as the average of the values obtained in the two sampling points in bathing waters

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