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Virus contamination and infectivity in beach environment: Focus on sand and stranded material

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ABSTRACT

To assess the exposure of beachgoers to viruses, a study on seawater, sand, and beach-stranded material was carried out, searching for human viruses, fecal indicator organisms, and total fungi. Moreover, for the first time, the genome persistence and infectivity of two model viruses was studied in laboratory-spiked sand and seawater samples during a one-week experiment. Viral genome was detected in 13.6 % of the environmental samples, but it was not infectious (Human Adenovirus – HAdV, and enterovirus). Norovirus and SARS-CoV-2 were not detected. The most contaminated samples were from sand and close to riverine discharges. In lab-scale experiments, the infectivity of HAdV5 decreased by ~1.5-Log_{10} in a week, the one of Human Coronavirus-229E disappeared in <3 h in sand. The genome of both viruses persisted throughout the experiment. Our results confirm viral contamination of the beach and suggest HAdV as an index pathogen for beach monitoring and quantitative risk assessment.

1. Introduction

Environmental circulation of virus is well-documented since 1940 in many matrices, e.g., surfaces, air, sediments, foods, waters. The epidemiological relevance of such matrices in the viral infections is widely confirmed through different exposure pathways (ingestion, inhalation, contact) (Labadie et al., 2020). Viruses are able to remain infectious under a wide range of environmental conditions, thus enhancing their transport to susceptible hosts (Pirtle and Beran, 1991). Coastal environments provide aggressive physicochemical factors (i.e., salinity, ultraviolet solar radiation, high temperature), but the viruses can persist in seawaters as demonstrated by worldwide monitoring studies (e.g., Wyn-Jones et al., 2011; Yang et al., 2012; Love et al., 2014; Rusiñol et al., 2015; Bonadonna et al., 2019) and their involvement in bathing water-related illnesses is also epidemiologically confirmed, showing that adenovirus, norovirus and coxsackieviruses are the most common causative viral agents (Sinclair et al., 2009; King et al., 2014; Graciaa et al., 2018). However, in a beach environment, water recreation and swimming are only one possible exposure pathway to pathogens, since beachgoers more often interact with sand owing to the longer time spent on the beach compared to the water, especially at higher latitudes (WHO, 2003, 2021). Beach sand hosts a huge variety of microorganisms (bacteria, fungi, parasites, and viruses): some of them belong to the autochthonous microbial communities of the sand ecosystem but can behave as opportunistic pathogens (e.g., fungi; Brandão et al., 2021), while others derive from humans and other warm-blooded animals. Human- or animal-related contamination of the sand occurs via water-borne transport through recreational waters that are affected by urban sewages or agricultural runoff (Whitman et al., 2014) or via direct deposition of fluids or excreta by beach visitors, including animals (Valério et al., 2022). Moreover, beach-stranded material can contribute to the microbial contamination of the sand as a result of the complex circulation of microorganisms in the waste-sand-water interface (Weiskerger et al., 2019; Federigi et al., 2022). Therefore, sand could act as a

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vehicle of biological agents through ingestion or contact as suggested by some epidemiological evidence that linked digging in beach sand to an increased risk of gastrointestinal illness (Bonilla et al., 2007; Heaney et al., 2009, 2012) or of skin diseases (Esiobu et al., 2013). In the last update of the guidelines for bathing waters, WHO reported a provisional value for fecal indicators on the beach based on accidental ingestion of intestinal enterococci (60 colony forming unit (CFU) per gram of sand; WHO, 2021). In addition, a threshold value of 89 CFU/g for total fungi has been calculated from a large dataset of samples of beach sand (Brandão et al., 2021). However, such guidelines values were not health risk-based, because of the lack of specific epidemiological studies, linking the symptoms with the concentration of beach-associated microbes. Health risk assessment on beaches still have research needs, including the choice of appropriate indicators, index pathogens, and the methods for their detection (Sabino et al., 2014; Solo-Gabriele et al., 2016), especially regarding the viruses. In fact, to date, little work has been done on the investigation of viral contamination of beach sand (Pianetti et al., 2004; Shah et al., 2011; Yamahara et al., 2012; Monteiro et al., 2016) despite the role of viruses in the epidemiology of recreational diseases. The recent severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic has raised interest and concern about the occurrence and persistence of viruses, especially coronaviruses, in marine environment (Mordecai and Hewson, 2020; Guo et al., 2021), although till now there was a paucity of data on the survival of coronaviruses in water matrices (Gundy et al., 2009). However, the virus persistence in beach sand still remains speculative with some inference (not experimentally driven) regarding the effects of ultraviolet (UV) radiation and heat on SARS-CoV-2 (Efstratiou and Tzoraki, 2021).

The aim of this work was to study the viral contamination of beach environment, in relation with the microbial contamination generally considered for safety assessment. Moreover, our study is aimed at filling the gaps of knowledge that currently hamper the design of a virological risk assessment for beachgoers. Therefore, specific objectives of the study refers to: (i) evaluate the type of microbial, and especially viral, contamination that can be found in the sand in a tourist beach; (ii) investigate the role of the matrix (bathing waters, beach-stranded material, and sand) and the location of the sampling points in the viral and microbial contamination; (iii) understand the relationship between the viral contamination and fecal indicator organisms (FIOs) or total fungal count; (iv) search for a FIO parameter that can be used for the estimation of the viral contamination; (v) estimate the persistence (infectivity and genome) of viruses in sand.

To address the above-mentioned topics, the contamination of a touristic beach located in the north-west Mediterranean has been studied by sampling seawater, beach-stranded material, and the sand beneath and focusing on FIOs, fungal count and viral pathogens. The infectivity and genome persistence in seawater and sand were also assessed through in vitro experiments using two model viruses: Human Adenovirus type 5 as representative of an unenveloped virus widely distributed in water matrices and commonly used as index pathogen for recreational waters (e.g., McBride et al., 2013; Federigi et al., 2020), and Human Coronavirus 229 E as a representative of enveloped viruses, often used as a surrogate for studying highly pathogenic coronaviruses (e.g., SARS-CoV-2) survival in various environmental matrices (Gundy et al., 2009; Carducci et al., 2020).

2. Materials and methods

2.1. Study location and sampling

The study location was the coastal area of the municipality of Pietrasanta (north-west Tuscany, Italy), that was chosen to represent a model for tourist beaches in the Mediterranean, impacted by land-based sources of contamination (Fig. 1A), here represented by two riverine discharges. Three sampling points were identified along the studied shoreline (4.8 km long): two sites close to the mouth of the watercourses that bordered the study area, namely the Motrone canal (Lat: 43.915 N; Long: 10.206 E) and the Fiumetto ditch (Lat: 43.936 N; Long: 10.187 E) and the third site in the middle (Lat: 43.924 N; Long: 10.197 E) (Fig. 1B).

A total of 12 monthly monitoring campaigns were carried out between August 2020 and August 2021, and three types of matrices were collected for each sampling point: seawater, beach-stranded material (hereafter stranded material), and the sand underneath such material (Table S1). The stranded material consisted of residues of terrestrial

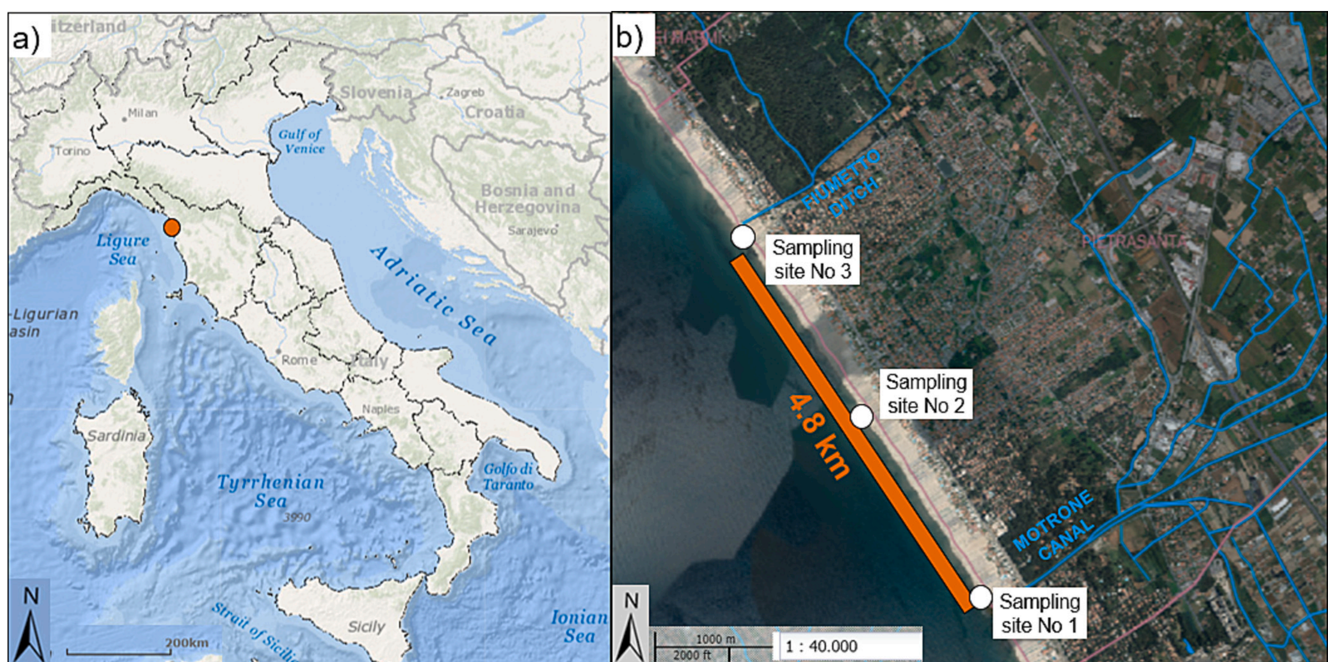


Fig. 1. Location of the study area. In (a) the orange dot indicates the study location in Italy (GISTAT, Italian National Statistical Institute, https://gisportal.istat.it). The area inside the circle from (a) is depicted in panel (b), with the main hydrology (GEOscopio WMS, Tuscany Regional Government, http://www502.regione.toscana.it/geoscopio/geologia.html) and the location of the sampling sites.

plants and seagrasses (beach cast), which were sometimes entangled in plastic debris (Menicagli et al., 2022). Sampling collection procedures are detailed below.

(i) *Seawater collection*. Seawater samples were taken from a water that is at least one meter deep and 30 cm below the water's surface, according to the rules on the handling of samples by EU (2006). Samples for analyses of human viruses and other parameters were collected separately and in different containers. For human virus analysis, 10 L samples of seawater were collected in tanks previously disinfected with sodium hypochlorite (5 % active chlorine) then neutralized with a 25 % sodium thiosulfate aqueous solution (Carducci et al., 2009). For FIO and total fungi analysis, 500 milliliter (mL) samples were collected in sterile bottles.

(ii) *Stranded material and sand collection*. One-hundred grams composite samples were collected from each sampling site using sterile gloves, separately for stranded material and the sand beneath. Briefly, in each site, three equidistant sub-samples of stranded material (approx. 1 m from each other) were collected along the strandline and then mixed together into a sterile plastic bag. Then, sand samples were taken aseptically from under the stranded material previously collected and placed in a separate sterile plastic container (Sabino et al., 2011). For the subsequent analysis, the stranded material was divided into fragments of <5 cm (where necessary).

In total, 88 samples were collected and analysed: 19 samples of seawater (samples were not collected during winter months and when the sea was rough), 36 of sand and 33 of stranded material (on one sampling date no residues were found on the beach) (see Table S1 for samples details).

2.2. Microbiological analysis of environmental samples

The seawater, sand and stranded material samples were analysed for:

(i) FIOs: total coliforms, *E. coli*, Intestinal Enterococci (hereafter enterococci), and somatic coliphages;
(ii) Total Fungal count (hereafter total fungi);
(iii) Human viruses: Human Adenovirus (HAdV), Norovirus genogroup II (NoV ggII), Enterovirus, and Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). This latter virus was included due to the pandemic and the lack of data regarding its presence in beach-related matrices.

The following limits of detection are derived from the analysed volumes and the method sensitivity.

2.2.1. Fecal indicator organisms

2.2.1.1. Seawater. Ten milliliters of seawater were analysed following the ISO 9308-3:1998 for total coliform/*E. coli* and the ISO 7899-1:1998 for enterococci, using the Colilert and Enterolert with Quanti-Tray (IDEXX Laboratories, Maine, USA). The limit of detection was 10 Most Probable Number (MPN)/100 mL (Sabino et al., 2011). For somatic coliphages, 20 mL of seawater were analysed directly according to the BS EN ISO 10705-2:2001 (double agar-layer method) on *E. coli* strain CN (ATCC 700078). The limit of detection was 5 Plaque Forming Unit (PFU)/100 mL (Yamahara et al., 2012).

2.2.1.2. Beach sand and stranded material. Samples of 50 g for each matrix, which were not dried to retain their natural water content, were eluted with 500 mL of sterile distilled water (1:10 ratio) and shaken for 30 min at 100 rpm (Sabino et al., 2011; Brandão et al., 2020). For bacterial parameters, the eluates (10 mL) were analysed using Colilert and Enterolert with Quanti-Tray (IDEXX Laboratories, Maine, USA) for the detection of total coliforms/*E. coli* and enterococci, respectively. The limit of detection was 1×10^2 MPN/100 g. For somatic coliphages, the eluate (300 mL), supplemented with $MgCl_2$, was filtrated through a 0.22-μm pore size cellulose nitrate filter. The membranes were then cut and eluted with 12 mL of 3 % beef extract at pH 9.0 (Yamahara et al., 2012). The eluate (10 mL) was then assayed according to the BS EN ISO 10705-2:2001, as previously indicated. The limit of detection was 3 PFU/100 g.

2.2.2. Total fungal count

2.2.2.1. Seawater. Samples of 0.1 mL, in triplicate, were poured onto Petri dishes containing Sabouraud Agar (SA) and incubated at 23 $\pm$ 1.0 °C for 5 days (ISS, 2007). The total fungi were obtained as the mean of the counts on the three replicated plates. The limit of detection was 3 $\times 10^2$ CFU/100 mL.

2.2.2.2. Beach sand and stranded material. Samples weighing 40 g, which were not oven-dried, were eluted with distilled water (1:1 ratio) as previously described for FIOs. Then, 0.1 mL of the eluate was poured, in triplicate, onto Petri dishes containing SA, as described above. The limit of detection was 3×10^2 CFU/100 g.

2.2.3. Human viruses

2.2.3.1. Sample preparation. Seawater samples were concentrated by two-step tangential flow ultrafiltration, using two different apparatus, both equipped with polysulphone membranes with 10 kDa molecular cutoff (Pall Europe, Banbury, UK) as previously described (Muscillo et al., 1997; Carducci et al., 2009). Briefly, 10 L samples were concentrated to 0.04 L through two subsequent ultrafiltration stages. At the end of each stage the membranes were washed with 3 % beef extract at pH 9, to recover the viral particles remained adsorbed on them and the washing liquid was neutralized to pH 7 and then added to the concentrated sample. Chloroform (1:10 by volume) was used for bacterial decontamination. To remove residual chloroform, the samples were shaken for 30 min, centrifuged at 1200 $\times g$ for 20 min, and the supernatant was recovered and aerated for 2 h.

For beach sand and stranded material, the samples preparation was the same as described for somatic coliphages (Sect. 2.2.1.2), namely it was based on agitation of each solid sample with sterile distilled water, filtration of the extraction fluid through 0.22-μm filter membranes, and elution of viral particles from the filters using 3 % beef extract. After the sample treatment, the virological analysis was carried out, first with biomolecular assays (real time (RT)-qPCR) and then by integrated cell culture (ICC)-(RT)PCR to assess infectivity of the positive samples.

2.2.3.2. Biomolecular analysis. The nucleic acids were extracted from 200 μL (for DNA) and 140 μL (for RNA) of the eluant fluids using QIAmp Viral DNA and RNA kits (Qiagen, Germany), respectively, according to the manufacturer's protocols. The genome of HAdV, NoV ggII, enterovirus, SARS-CoV-2, and Human Coronavirus 229E (HCoV229E) were then searched by real time (RT)-qPCR on TaqMan chemistry. All reactions were performed in triplicate on 96-well optical plates using an ABI 7300 sequence detector system (Applied Biosystems, Foster City, California). Real time qPCR was performed using Taq Man Universal Master Mix (Applied Biosystems) for HAdV and AgPath-ID™ One-Step RT-PCR Reagents (Life Technologies) for RNA viruses. The list of primers and probes and their concentrations is reported in Table 1, as well as the protocol conditions.

In solid samples, the limit of detection was 8.6×10^2 GC/100 g for HAdV and 2.9×10^2 GC/100 g for RNA viruses, whereas in water samples, it was 1.0×10^3 GC/10 L and 1.8×10^2 GC/10 L, respectively.

Table 1
Oligonucleotide primers and probes used for the viral detection by (RT)qPCR. Target regions are also reported for each viral parameter.

Primer or probe names	Concentration (μM)	Sequence (5′-3′)	Thermal cycle	Reference
Human adenovirus (hexon gene)				
Primer AdF	0.9	CWTACATGCACATCKCSG G	50 °C: 2 min, 95 °C: 10 min, 45 cycles (95 °C: 15 s; 60 °C: 1 min)	Hernroth et al., 2022
Primer AdR	0.9	CRCGGGCRAAYTGCACCA G		
Probe AdP	0.225	FAM-CCGGGCTCAGGTACTCCGA GGCGTCCT-TAMRA		
Norovirus genogroup II (RdR Pol gene)				
Primer JJV2F	1	CAAGAGTCAATGTTTAGGTGGATGAG	48 °C: 30 min, 95 °C: 10 min, 45 cycles (95 °C: 15 s; 60 °C: 1 min)	Skraber et al., 2009
Primer COG2R	1	TCGACGCCATCTTCATTCACA		
Probe RING2-TP	0.1	FAM-TGGGAGGGCGATCGCAA TCT-BHQ		
Enterovirus (5′ UTR region)				
Primer EVF	0.6	GGCCCCTGAATGCGGCTAAT	48 °C: 30 min, 95 °C: 10 min, 45 cycles (95 °C: 15 s; 60 °C: 1 min)	Donaldson et al., 2002
Primer EVR	0.6	CACCGGATGGCCAATCCAA		
Probe EV	0.25	FAM-CGGACACCCAAAGTAGT CGGTTCCG-TAMRA		
SARS-CoV-2 (ORF1ab)				
2297-CoV-2-F	0.5	ACA TGG CTT TGA GTT GAC ATC T	50 °C: 30 min, 95 °C: 5 min, 45 cycles (95 °C: 15 s; 60 °C: 30s)	La Rosa et al., 2021
2298-CoV-2-R	0.9	AGC AGT GGA AAA GCA TGT GG		
2299-CoV-2-P	0.25	FAM-CAT AGA CAA CAG GTG CGC TC-MGBEQ		
Human coronavirus 229E (ORF1ab)[a]				
2288-HCV 229E F1	0.5	GAT GCA ACT ACA GCC TAC GC	50 °C: 30 min, 95 °C: 5 min, 45 cycles (95 °C: 15 s; 60 °C: 30s)	La Rosa et al., 2021
2289-HCV 229E R1	0.9	AGT TAA CGC TCA AAA CGC AAT		
2290-HCV 229E P1	0.25	FAM-TTT CAG GCT GTA AGT TCT AAC ATT-TAMRA		

[a] Only for in vitro experiments.

The extracted nucleic acid from environmental samples with positive (RT)-qPCR signals were then analysed by qualitative nested (RT)-PCR and according to published protocols for HAdV (Allard et al., 1992) and enterovirus (Gilgen et al., 1997). The PCR products were sequenced through Sanger sequencing from GATC service (Eurofins Genomics, Germany). Sequence analysis was performed with the NCBI GenBank.

2.2.3.3. Integrated cell culture (RT)PCR. The environmental samples with positive (RT)-qPCR signals for HAdV and enterovirus were tested for infectivity using the ICC-(RT) PCR technique, to speed up the infectivity test (Ryu et al., 2018). The cell lines used were A549 cells (ATCC CCL-185) for HAdV and LLC-MK2 cells (ATCC CCL-7) for enterovirus. These were grown in 75-cmq flasks, maintained in Eagle's minimum essential medium (EMEM) containing 10 % fetal bovine serum (FBS), 10 % L-glutamine, and 0.125 % gentamycin, and incubated at 37 °C with 5 % CO_2. The inoculation procedures were performed in 25-cmq flasks containing a cell monolayer that was 24–48 h old and 90 % confluent, according to American Type Culture Collection recommendations (ATCC, 2016). The liquid inoculum was represented by the eluant fluid obtained from Sect. 2.2.3.1 and corresponding to the (RT) qPCR-positive sample (Sect. 2.2.3.2). A negative control was prepared, adding only EMEM to the cells' monolayers. After a one-hour contact period between the liquid inoculum and the cell monolayer, EMEM supplemented with 2 % FBS was added to the cell monolayer and flasks were observed daily for five days to detect the cytopathic effect (CPE). Irrespectively of any evidence of a cytopathic effect, the flasks were then submitted to three freeze-thaw cycles, to induce the virus release from cells, cell debris was pelleted with centrifugation at 160 RCF (x g) for 3 min, and supernatants were processed for genome extraction and (RT) PCR: positive results were considered representative for viral replication (Fongaro et al., 2013).

2.3. Lab scale experiments on viral infectivity and genome persistence

2.3.1. Spiking viruses and cell cultures

The viruses and the cell lines permissive for their replication were obtained from the ATCC: namely, HAdV5 (ATCC VR-5) was propagated on A549 cells, and Alphacoronavirus HCoV-229E (ATCC VR-740) on the human lung fibroblast MRC-5 cell line (ATCC CCL-171). The viral suspensions used for experiments had a titer of 1×10^6 $TCID_{50}$/mL and 2.33×10^9 GC/mL for HAdV5 and 2.38×10^5 $TCID_{50}$/mL and 3.27×10^{14} GC/mL for HCoV229E. Stock viral suspensions were stored at — 80 °C until use.

2.3.2. Experimental design

The survival experiments were carried out on seawater and sand samples collected from the study site, both the real samples and after heat treatment using autoclave. All samples were preliminarily screened for the presence of HAdV and HCoV229E genomes, bacterial FIOs, and total fungi. Moreover, possible cytotoxic effect of natural microflora was tested by seeding cells (A549 and MRC-5 monolayers) with seawater and sand eluant (obtained as described in Sect. 2.2.3.1) at various dilutions in antibiotic-supplemented EMEM: 1:1 (undiluted), 1:2, 1:4, 1:8, 1:10. Microbiological analyses revealed the absence of HAdV and HCoV229E genomes as well as bacterial FIOs in the analysed samples, but high total fungi level in unautoclaved sand (7.3×10^3 CFU/100 g). Probably due to this high fungal level, all the tested dilutions of the unautoclaved sand eluant were found toxic for the cell monolayers, whereas no cytotoxicity was observed at 1:10 dilution for the other tested matrices. Therefore, further experiments were carried out only on autoclaved sand sample, and also seawater (both autoclaved and not autoclaved). The flowchart of the persistence experiments is reported in Fig. S1. For seawater, the pH was previously stabilized at neutrality with 10 mM HEPES buffer (Poulson et al., 2016). Then, separately for each virus suspension, aliquots of 100-μL were inoculated into Eppendorf tubes containing 900-μL of seawater and briefly mixed for homogenization: in total there were 21

aliquots for the autoclaved seawater and 21 for the unautoclaved one.

For autoclaved sand, separately for each virus suspension, aliquots of 300-μL were inoculated into Eppendorf tubes containing 1 g of dry sand (21 aliquots in total). The volume of liquid was previously defined to allow the complete wetting of sand.

After the viral spiking, aliquots were analysed at intervals starting from time zero and after 3 h, 6 h, 1 day, 2 days, 3 days, and 7 days. Each assay was performed in triplicate. The tubes were covered in aluminum foil to prevent exposure to light and then stored under controlled room temperature conditions (21 °C) and 50 % relative humidity. At the defined times, the samples were frozen at −80 °C until their analysis (Gundy et al., 2009).

2.3.3. Sample preparation and analysis

Spiked water samples were directly analysed with both molecular and cultural assays. Spiked sand samples were eluted with an equal volume of EMEM (Staggemeier et al., 2015) at pH 9, then centrifugated at 10,000 ×*g* for 10-min, and the supernatant was recovered adjusting its pH to neutrality and used for the subsequent analysis.

2.3.3.1. Molecular analysis. Genome extraction and real time (RT)-qPCR were performed as described in Sect. 2.2.3.2 for HAdV5 and HCoV229E, and using protocols as specified in Table 1. The limit of detection was 2.6×10^{1} GC/mL and 3.0×10^{3} GC/mL for HAdV5 and HCoV229E, respectively.

2.3.3.2. Infectivity assay. The viral infectivity was quantified through microtiter endpoint titration in 96-wells polystyrene plates and using the Spearman-Karber formula (Ramakrishnan, 2016) for the calculation of the viral titer. Briefly, 0.5-mL of virus-inoculated sample was diluted tenfold by adding EMEM supplemented with 10 % L-glutamine and 0.125 % gentamycin. Serial dilutions started from 10^{-1} to 10^{-4}, since the 1:10 dilution was nontoxic on the cell monolayers (Sect. 2.3.2). Each virus dilution (75 μL) was seeded into six wells of the microtiter plate, containing 75 μL of EMEM prepared as above, and 50 μL of the appropriate cell suspension (approx. 10^{6} cells/mL of A549 or MRC-5). A negative control was also prepared, by adding a double volume of EMEM to the cell suspension (both for A549 and MRC-5 cells). Plates were covered and incubated at 37 °C under 5 % CO_2 for 5 days, and then examined for cytopathic effects. The limit of detection was 1.3×10^{1} 50 % tissue culture infective dose ($TCID_{50}$)/mL.

2.4. Statistical analysis

All statistical analyses and graphical representations were performed using R v. 4.1.3, with its specific packages as reported below. Data were presented using descriptive statistics, including frequency of positive samples and means with standard deviations of concentration data. For the statistical analysis, microbial concentrations were Log_{10} transformed to reduce variability in the environmental data and samples below the limit of detection were assigned a value that was half of the lowest detection limit (Heaney et al., 2014). The microbial data distributions for each matrix and parameter were tested with the Shapiro-Wilk normality test and the Skewness statistic (package *moments*), and the results supported the use of the parametric approach for the statistical analysis. The influence of the environmental matrix (seawater, beach sand, and stranded material) and sampling point (points No1, No2, and No3) on the distribution of microbial parameters were evaluated using two-way ANOVA. The association between parameters, divided by the matrix, was measured by the Pearson correlation coefficient (r), and shown as a correlation matrix (package *corrplot*).

The differences in average density of Log_{10} FIOs between the HAdV positive and negative samples were assessed using the unpaired Student's *t*-test. The probability of a HAdV-positive outcome was estimated from the FIO concentrations through logistic regression models, as described by Wyer et al. (2012) for recreational waters.

Values of $p \leq 0.05$ were considered as statistically significant, but also small departures from significance were presented and discussed.

In the in vitro experiments, the viral abatement (infectious titer or genomic copies) was expressed as a logarithmic reduction (LR), which was calculated as $LR = Log_{10}(N_t/N_0)$, where:

- N_0 is the estimated viral titer in the spiked sample, according to the formula: $SpC \times SpV / SaV$ (where *SpC* and *SpV* are the titer and the volume of the added viral suspension, respectively, and *SaV* is the volume of the spiked sample).
- Nt is the viral titer measured at a time t.

3. Results

3.1. Beach microbial contamination

Fig. 2 shows the percentages of positive samples for microbial parameters, in each matrix. In seawaters, total coliforms were detected in 100 % of samples, followed by *E. coli* and somatic coliphages (89 %), enterococci (78 %), fungi (74 %), and human virus genome (5.3 %). Stranded material was mostly contaminated by total coliforms (94 %), followed by fungi (91 %), enterococci (70 %), *E. coli* (67 %), somatic coliphages (52 %) and the human virus genome (12 %).

On the other hand, in sand, the most frequently detected microbial parameters were fungi (89 %), followed by total coliforms (83 %), enterococci (61 %), *E. coli* (53 %), somatic coliphages (31 %) and human virus genome (19 %).

Considering specific viruses, HAdVs were the most frequently detected (13.6 % of total samples), mostly in sand, followed by stranded material, whereas only one sample was positive in seawater (Table 2). The enterovirus genome was detected twice, only in sand, and in association with HAdV. Neither NoV ggII nor SARS-CoV-2 genomes were detected. Regarding infectivity assays, no positive results were observed with ICC-(RT)PCR on samples containing viral genome.

The viral strains, identified by sequencing are reported in detail in Table S2, separately for each sampling point. In summary, the HAdV strains belonged to species F (serotype 41) and C (serotypes 1, 2, and 6). Serotype 2 was the most frequently detected (41 % of the 12 positive samples), followed by HAdV41 (33 %). The strains of enterovirus identified were assigned to types of EV-A species (A71 and A90).

The data on microbial concentrations over the whole study period are shown in Fig. S2, while their geometric means and standard deviations are summarized in Table 2. This calculation was possible because the Log_{10} concentration values of each microbial parameter, separately for each matrix, showed a good similarity to the normal distribution ($p > 0.05$ for Shapiro-Wilk tests and/or skewness statistic ranging from −1 and 1, Table S3).

The abundance (and the frequencies) of microbial parameters according to sampling points and environmental matrices are reported in Table S4. Overall, stranded material was significantly more contaminated than the other matrices for bacterial FIOs and total fungi (two-way ANOVA, $p < 0.01$).

The location of the sampling point influenced the levels of FIOs with the site far from the river mouths (point No2) less polluted than those located at the riverine discharges (points No1 and No3), and such differences was close to statistical significance for total coliform (two-way ANOVA, $p = 0.052$) and coliphages (two-way ANOVA, $p = 0.091$). Regarding human viruses, the small number of positive samples hampered the statistical analysis, but their occurrence showed a similar trend influenced by matrix and point: most of the viral pathogen-positive samples were detected in sand (58.3 %, 7/12) and stranded material (33.3 %, 4/12), and in sampling points located at the river mouths (83.3 %, 10/12) (Table S4).

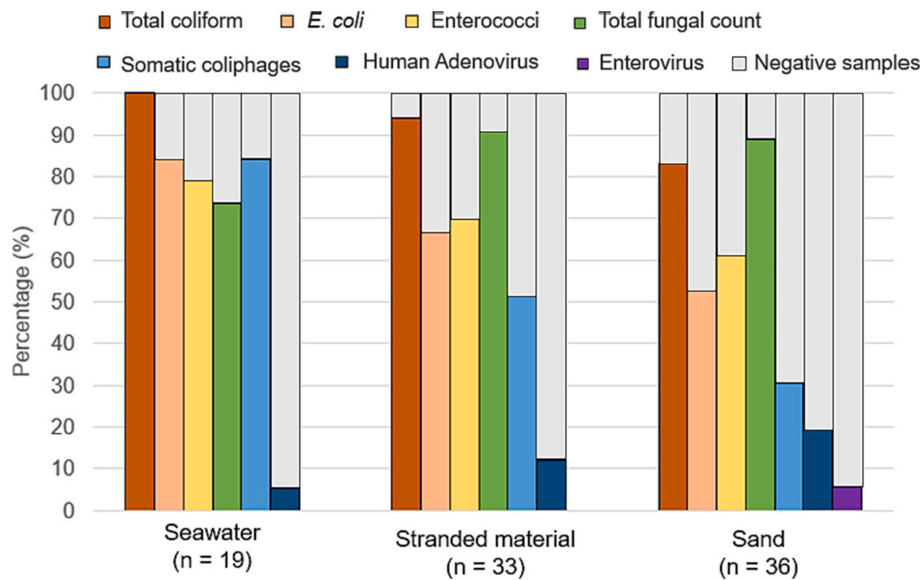


Fig. 2. Occurrence of microbiological parameters in each matrix (seawater, sand, and stranded material).

Table 2
Summary statistics for FIOs, total fungi and human virus (HAdV and enterovirus) concentrations, separately for each environmental matrix (data from different sampling points were combined).

Microbial parameter	Positive samples n° (%)	Geometric mean ± standard deviation
Seawater (*n* = 19)		
Total coliforms (MPN/100 mL)	19 (100)	$4.16 \times 10^2 \pm 6.30$
E. coli (MPN/100 mL)	16 (84.2)	$7.87 \times 10^1 \pm 8.8$
Intestinal Enterococci (MPN/100 mL)	15 (78.9)	$5.64 \times 10^1 \pm 9.53$
Somatic coliphages (PFU/100 mL)	16 (84.2)	$2.96 \times 10^1 \pm 7.44$
Total fungi (CFU/100 mL)	14 (73.7)	$6.20 \times 10^2 \pm 2.73$
HAdV (GC/10 L)	1 (5.3)	8.54×10^3 (single value)
EV (GC/10 L)	Not detected	NA
Stranded material (*n* = 33)		
Total coliforms (MPN/100 g)	31 (93.9)	$1.26 \times 10^4 \pm 16.8$
E. coli (MPN/100 g)	22 (66.7)	$8.53 \times 10^2 \pm 23.8$
Intestinal Enterococci (MPN/100 g)	23 (69.7)	$1.15 \times 10^3 \pm 20.9$
Somatic coliphages (PFU/100 g)	17 (51.5)	$4.75 \times 10^0 \pm 4.03$
Total fungi (CFU/100 g)	30 (90.9)	$1.74 \times 10^4 \pm 8.23$
HAdV (GC/100 g)	4 (12.1)	$1.06 \times 10^3 \pm 5.36$
EV (GC/100 g)	Not detected	NA
Beach sand (*n* = 36)		
Total coliforms (MPN/100 g)	30 (83.3)	$1.26 \times 10^3 \pm 11.2$
E. coli (MPN/100 g)	19 (52.8)	$1.74 \times 10^2 \pm 4.89$
Intestinal Enterococci (MPN/100 g)	22 (61.1)	$2.87 \times 10^2 \pm 7.51$
Somatic coliphages (PFU/100 g)	11 (30.6)	$2.49 \times 10^0 \pm 3.04$
Total fungi (CFU/100 g)	32 (88.9)	$4.06 \times 10^3 \pm 5.09$
HAdV (GC/100 g)	7 (19.4)	$1.33 \times 10^3 \pm 4.38$
EV (GC/100 g)	2 (6.1)	3.63×10^2 and 1.97×10^4

CFU = colony forming unit; GC = genomic copies; MPN = most probable number; PFU = plaque forming unit; HAdV = human adenovirus, EV = enterovirus, NA = not applicable.

3.2. Correlations between microbial parameters

The correlations between the microbial parameters are shown in Fig. 3, separately for each environmental matrix, and reported in Table S5. Although all parameters appeared to be positively correlated, the strength of correlation differed depending on the environmental matrix. In seawater (Fig. 3a), all bacterial FIOs were highly and statistically inter-correlated, and also the somatic coliphages showed a high correlation with the bacterial indicators, namely total coliforms (r = 0.881, $p < 0.0001$), *E. coli* (r = 0.768, $p < 0.0001$), and enterococci (r = 0.649, $p < 0.0001$). In contrast, the total fungi did not correlate significantly with the FIOs. The correlations for HAdV were not calculated because only one sample was positive.

In the stranded material (Fig. 3b), the correlations were statistically significant among bacterial FIOs, although the level of association was lower than seawater. Coliphages exhibited a significant correlation only with total coliforms (r = 0.511; $p < 0.01$) and *E. coli* (r = 0.522, $p < 0.001$), and total fungi with enterococci (r = 0.445; $p < 0.05$). HAdV showed a slight (not significant) correlation with *E. coli* and enterococci. In the sand (Fig. 3c), bacterial FIOs and total fungi correlations were similar to stranded material, while somatic coliphages showed no correlation and HAdV showed a slight, although not significant, correlation with total coliforms.

This association was confirmed by the *t*-test comparison between FIOs concentrations in HAdV positive and negative samples (12 and 76 respectively) (Fig. S3). Overall, these concentrations were higher in HAdV-positive samples, however the difference was statistically significant only for total coliforms (*t*-test, $p < 0.05$).

In order to obtain a FIO predictor of viral contamination of sand, we used logistic regression to model the probability of an HAdV-positive sand sample using the FIO concentration data as continuous variables. In agreement with the previous results, total coliforms were found to be the best predictors (p = 0.06) for HAdV genomic copies. Their distribution was thus used to estimate the probability of HAdV positivity (Fig. 4), which was low (< 5 %) when the total coliforms concentration was <100 MPN/100 g but increased rapidly above this value.

3.3. Virus survival experiments

The results of the infectivity and genome persistence are reported in Tables 3 and 4. Images of the infected cells and their typical alterations are showed in Fig. S4. The HAdV5 infectivity and genome were detected

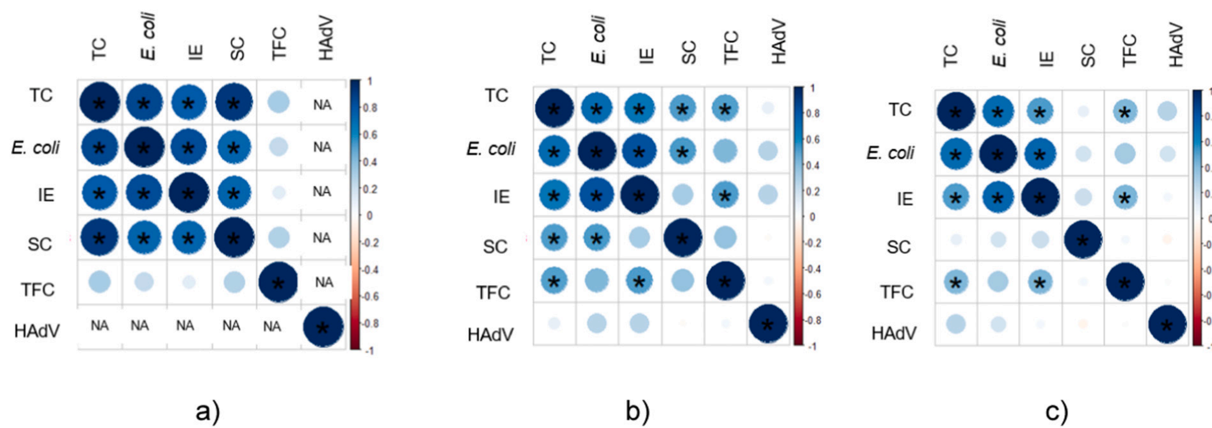
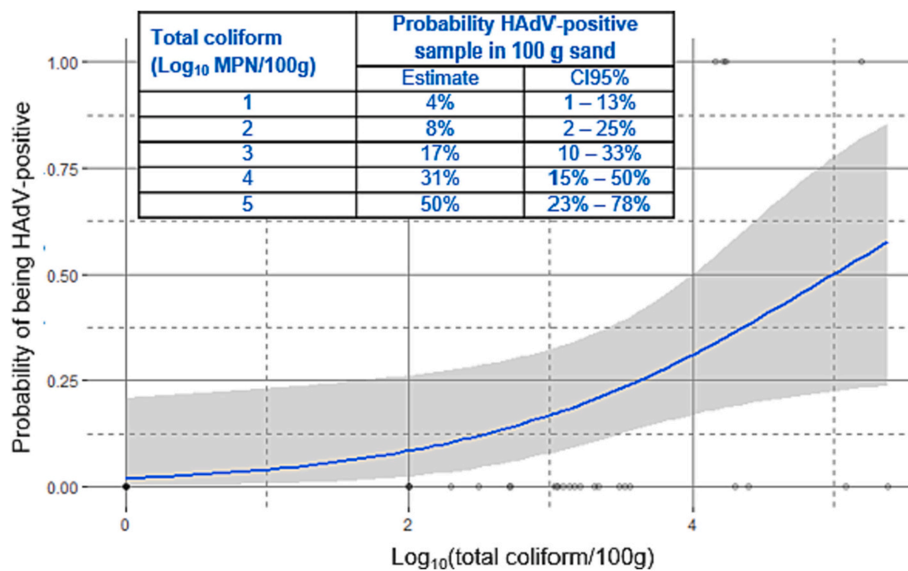


Fig. 3. Correlations between various microbial indicator parameters in seawater (a), stranded material (b), and sand (c). Colors represent the value of Pearson's r correlation: the darker the color, the larger the correlation magnitude. High correlation values are for $r \geq 0.5$ and $r \leq -0.5$ for direct and indirect relationships, respectively (dark colors), while $-0.5 < r < 0.5$ represent low correlations (light colors). Statistically significant correlations are highlighted by asterisks. TC = Total Coliform; IE = Intestinal Enterococci; SC = Somatic Coliphages; TFC = Total Fungal Count; HAdV = Human Adenovirus; NA = not applicable, since only one sample was positive for HAdV in seawater.



Total coliform (Log_{10} MPN/100g)	Probability HAdV-positive sample in 100 g sand	
	Estimate	CI95%
1	4%	1 – 13%
2	8%	2 – 25%
3	17%	10 – 33%
4	31%	15% – 50%
5	50%	23% – 78%

Fig. 4. Logistic regression models predicting the probability of HAdV in 100 g from Log_{10} total coliform concentrations (per 100 g) in sand samples. CI 95 % = 95 % confidence interval.

for the entire duration of the experiments. The reduction in viral infectivity was quite similar in the three matrices, with a global decay of 1.03 ± 0.17 Log_{10} for seawater, 0.93 ± 0.25 Log_{10} for autoclaved seawater, and 1.67 ± 0.19 Log_{10} for autoclaved sand. On the other hand, the genome persistence after seven days showed some matrix-dependent differences: in the autoclaved seawater, the genome reduction was 0.25 ± 0.01 Log_{10}, which in the seawater was 0.48 ± 0.04 Log_{10}, while in the sand it reached 2.48 ± 0.45 Log_{10}. Infectious HCoV229E was no longer detectable after three days in autoclaved seawater, after 3 h in seawater, and after time zero in sand. On the other hand, the genome persisted for all the experiments: after seven days, HCoV229E was reduced by 0.57 ± 0.89 Log_{10} in autoclaved seawater, 0.78 ± 0.32 Log_{10} in seawater, and 1.90 ± 0.09 Log_{10} in autoclaved sand.

4. Discussion

The results of our study show a moderate contamination of beach sand by bacterial FIOs, in agreement with other studies (Halliday and Gast, 2011; Whitman et al., 2014). The levels of enterococci we detected (except for two samples) were below the proposed WHO threshold of 6.000 CFU/100 g. In addition, the FIO contamination, which was clearly linked to the riverine discharges, decreased from seawater to stranded material and to sand as already reported by the few papers that have studied the same matrices (Englebert et al., 2008; Imamura et al., 2011). Focusing on the human viruses, we observed an analogous link with rivers, but an increase in the pollution from seawater to sand, suggesting a possible concentration effect on the virus by solid material. On the other hand, the fungal contamination was similar in the three sampling points, in agreement with its environmental origin, and higher in the stranded material, which are ideal conditions for fungi to multiply (e.g., nutrient availability, moisture) as reported by Weiskerger et al. (2019).

Our data on the viral contamination of beach sand confirm the presence of the enterovirus and adenovirus, as also reported by the few studies available on the same material (Pianetti et al., 2004; Shah et al., 2011; Monteiro et al., 2016). In particular, we found similarities with the study carried out by Monteiro et al. (2016) along Portugal beaches, in terms of absence of NoV ggII genome and of presence of HAdV with the highest frequency among the searched viruses (they also found

Table 3
Results of the survival of infectivity and genome persistence experiments of HAdV5 in the three matrices.

Time	Seawater	Autoclaved seawater	Autoclaved sand
Infectivity ($TCID_{50}$/mL or $TCID_{50}$/g)			
Expect. Conc.	1×10^5	1×10^5	3.33×10^5
Time zero	$8.87 \times 10^4 \pm 2.04$	$4.24 \times 10^5 \pm 1.00$	$1.34 \times 10^5 \pm 1.47$
3 hours	$8.87 \times 10^4 \pm 2.04$	$4.24 \times 10^5 \pm 1.00$	$1.34 \times 10^5 \pm 1.47$
6 hours	$7.07 \times 10^4 \pm 3.23$	$1.04 \times 10^5 \pm 1.25$	$9.13 \times 10^4 \pm 1.47$
1 day	$5.47 \times 10^4 \pm 4.72$	$8.03 \times 10^4 \pm 1.25$	$6.22 \times 10^4 \pm 1.47$
2 days	$1.52 \times 10^4 \pm 1.25$	$2.89 \times 10^4 \pm 1.72$	$1.18 \times 10^4 \pm 4.72$
3 days	$1.04 \times 10^4 \pm 1.25$	$1.34 \times 10^4 \pm 2.76$	$1.04 \times 10^4 \pm 1.25$
7 days	$9.13 \times 10^3 \pm 1.47$	$1.18 \times 10^4 \pm 1.80$	$7.07 \times 10^3 \pm 1.56$
Genome (GC/mL or GC/g)			
Expect. Conc.	2.33×10^8	2.33×10^8	7.78×10^8
Time zero	$1.44 \times 10^8 \pm 1.10$	$1.40 \times 10^8 \pm 1.02$	$3.36 \times 10^7 \pm 1.11$
3 hours	ND	ND	ND
6 hours	ND	ND	ND
1 day	ND	ND	ND
2 days	ND	ND	ND
3 days	$1.14 \times 10^8 \pm 1.09$	$1.32 \times 10^8 \pm 1.03$	$7.39 \times 10^6 \pm 1.42$
7 days	$7.70 \times 10^7 \pm 1.09$	$1.30 \times 10^8 \pm 1.01$	$2.60 \times 10^6 \pm 2.79$

"Expect. Conc." refers to the expected virus concentration in the spiked samples. ND = not determined. Results are reported as geometric mean (10^x, where X is the mean of log_{10} transformed values) ± standard deviation of log_{10} transformed concentrations.

Table 4
Results of the survival of infectivity and genome persistence experiments of HCoV229E in the three matrices.

Time	Seawater	Autoclaved seawater	Autoclaved sand
Infectivity ($TCID_{50}$/mL or $TCID_{50}$/g)			
Expect. Conc.	2.38×10^4	2.38×10^4	7.93×10^4
Time zero	$2.38 \times 10^1 \pm 1.78$	$3.18 \times 10^1 \pm 1.33$	$4.94 \times 10^3 \pm 1.70$
3 hours	$1.47 \times 10^1 \pm 1.18$	$1.97 \times 10^1 \pm 1.55$	$<1.30 \times 10^1$
6 hours	$<1.30 \times 10^1$	$1.97 \times 10^1 \pm 1.55$	$<1.30 \times 10^1$
1 day	$<1.30 \times 10^1$	$1.79 \times 10^1 \pm 1.33$	$<1.30 \times 10^1$
2 days	$<1.30 \times 10^1$	$1.55 \times 10^1 \pm 1.23$	$<1.30 \times 10^1$
3 days	$<1.30 \times 10^1$	$1.47 \times 10^1 \pm 1.18$	$<1.30 \times 10^1$
7 days	$<1.30 \times 10^1$	$<1.30 \times 10^1$	$<1.30 \times 10^1$
Genome (GC/mL or GC/g)			
Expect. Conc.	3.27×10^{13}	3.27×10^{13}	1.09×10^{14}
Time zero	$5.04 \times 10^{13} \pm 2.31$	$4.98 \times 10^{13} \pm 1.46$	$4.77 \times 10^{13} \pm 2.48$
3 hours	ND	ND	ND
6 hours	ND	ND	ND
1 day	ND	ND	ND
2 days	ND	ND	ND
3 days	$1.99 \times 10^{13} \pm 3.30$	$3.13 \times 10^{13} \pm 1.70$	$6.44 \times 10^{12} \pm 1.33$
7 days	$5.43 \times 10^{12} \pm 2.10$	$8.78 \times 10^{12} \pm 7.84$	$1.36 \times 10^{12} \pm 1.24$

"Expect. Conc." refers to the expected virus concentration in the spiked samples. ND = not determined. Results are reported as geometric mean (10^x, where X is the mean of log_{10} transformed values) ± standard deviation of log_{10} transformed concentrations.

hepatitis A virus, that has not been searched in the present study). In our study, the detection of HAdV41 genome is not surprising due to its common association with sewage-related contamination (Fong et al., 2010; Ogorzaly et al., 2015). The lack of infectivity in our samples may be partially explained by the difficulty of replicating such HAdV strain in cell cultures (Jothikumar et al., 2005). From an epidemiological point of view, this type of HAdV has been recently associated with acute hepatitis in children (Karpen, 2022), although this hypothesis needs to be confirmed. However, the possible ingestion of sand containing this virus could represent a risk at least of gastroenteritis, since enteric strain of HAdV, as well as other enteric viruses, are able to infect host at very low doses. To estimate the health risk attributable to accidental ingestion of sand, a quantitative microbial risk assessment (QMRA) should be carried out, considering the infectious HAdV concentration in sand, the amount of sand that is unintentionally ingested, and the HAdV dose-response relationship. Such estimation goes behind the aim of the present paper, but a roughly estimate can be done assuming that the ratio between infectivity and genome was 1:100, according to our experimental results. Using the highest amount of HAdV detected in sand (3.38×10^2 GC/g, Table S4), we could estimate 3 infectious HAdV per gram, that could represent the daily exposure dose of children who accidentally ingest 1 g of sand per day (WHO, 2021). We can then apply this exposure estimate to the dose-response relationship derived by Teunis et al. (2016) from data of clinical trials, who found one case of gastroenteritis in every 100 exposures after the ingestion of a mean of 20 HAdV particles. With these assumptions, the estimated gastrointestinal risk in our beach scenario could be considered <1 %.

Among the other searched viruses, enterovirus was found only in two sand samples, both also contaminated by HAdV, while NoVs and SARS-CoV-2 were not detected. During the study period (February 2021–August 2021), SARS-CoV-2 genome was frequently (44 % positivity) detected in sewages entering a treatment plant of a nearby city (Carducci et al., 2022), that was monitored in the context of the Wastewater Based Epidemiology according to the indication of EU Commission (EU, 2021). Nevertheless, SARS-CoV-2 absence in seawater, stranded material and sand is in agreement with the low resistance of the coronaviruses in water environments (Carducci et al., 2020). This was also confirmed by the lab-scale experiments carried out in this paper, showing not only a very rapid decrease of coronavirus infectivity but also a decrease of its genome in both seawater and sand.

The data from our spiked-seawater samples showed that HAdV5 remained infective for a long time, as also reported in the literature (e.g., Enriquez et al., 1995; de Abreu Corrêa et al., 2012; Liang et al., 2017), whereas HCoV229E exhibited a rapid inactivation, in accordance with a recent lab-scale study on SARS-CoV-2 (Sala-Comorera et al., 2021). To the best of our knowledge, this is the first time that viral persistence has been tested in sand under controlled laboratory conditions (dark, 21 °C and 50 % humidity), showing the ability of HAdV to survive and a rapid loss of infectivity of HCoV229E. In real condition, beach sand exposed to sunlight, heat, UV radiation and drying could lead to a more rapid viral elimination; however, in wet sand and under the stranded material, the viral persistence could be quite long.

Our findings from the field and the laboratory studies show that HAdV is the most frequently detected, it maintained infectivity for long time in lab-scale spiked sand samples and it was (although weakly) related with total coliforms. This virus is also quite easy to detect both with biomolecular and culture methods and its use as index viral pathogen has been suggested for seawater (McBride et al., 2013; Wyer et al., 2012). However, the complexity of virological detection in environmental matrices has so far discouraged the use of HAdV as a routine monitoring parameter, even in seawater (Wyn-Jones et al., 2011). Therefore, estimating HAdV contamination from the concentration of bacterial indicators is very helpful to assess the risk of infection (WHO, 2016). In our study, we did not find significant correlations between FIOs and HAdV, except for total coliforms, probably due to the higher number of samples with both microbial parameters. The total coliforms parameter was thus used to predict the probability of viral positivity in 100 g of sand, indicating a threshold of 100 MPN/100 g for a presence of HAdV over 4 %. With this relation we could estimate the viral contamination from the value of an indicator easy to measure (WHO, 2016), thus allowing to implement QMRA for infective health risk from beach sand, that is a topic rarely addressed in the scientific literature (Shibata and Solo-Gabriele, 2012; Weiskerger and Brandão, 2020).

5. Conclusions

Our study strongly supports the need to include sand quality measures in regulatory programmes, also considering viral contamination and the role of stranded material. The main findings of the present work

can be summarized as follows:

1) The viral contamination of sand in a tourist beach was detectable, although limited to the HAdV and enterovirus genome.
2) The viral contamination detected in sand is higher than that in stranded material and seawater, and most of the viruses were detected close to the riverine discharges.
3) The viral (HAdV) contamination is related to FIOs, but significantly only for total coliforms and is unrelated to total fungi.
4) HAdV contamination can be estimated from the total coliforms.
5) The virus's ability to persist in sand differs depending on the virus and the analytical target: infectivity disappeared after 3 h for the coronavirus but was only slightly reduced after a week for the adenovirus.

Nevertheless, further research is needed to confirm the results obtained in the present paper and to improve the body of knowledge on the human health risk in the beach environment. Specific recommendations include:

- increasing space and time of the beach surveys, also including other pathogens possibly related to epidemiological studies;
- standardization and validation of the analytical methods for the virological analysis to make the results comparable;
- evaluation of environmental factors (e.g., light, temperature, relative humidity, salinity) on the presence and persistence of viruses in sand, also taking into account their viability in such matrix.

CRediT authorship contribution statement

Annalaura Carducci: Conceptualization, Methodology, Supervision, Funding acquisition, Writing – original draft. **Ileana Federigi:** Methodology, Investigation, Formal analysis, Writing – original draft. **Elena Balestri:** Writing – review & editing. **Claudio Lardicci:** Writing – review & editing. **Alberto Castelli:** Writing – review & editing. **Ferruccio Maltagliati:** Writing – review & editing. **Hongrui Zhao:** Investigation. **Virginia Menicagli:** Writing – review & editing. **Rossella Valente:** Investigation. **Davide De Battisti:** Writing – review & editing. **Marco Verani:** Writing – review & editing.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marpolbul.2022.114342.

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