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ORIGINAL PAPER

Detection of Human Enteric Viruses in French Polynesian Wastewaters, Environmental Waters and Giant Clams

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Abstract

Lack of wastewater treatment efficiency causes receiving seawaters and bivalve molluscan shellfish to become contaminated, which can lead to public health issues. Six wastewater samples, five seawater samples and three batches of giant clams from Tahiti (French Polynesia) were investigated for the presence of enteric viruses, but also if present, for the diversity, infectivity and integrity of human adenoviruses (HAdV). Enteroviruses (EV), sapoviruses (SaV) and human polyomaviruses (HPyV) were detected in all wastewater samples. In decreasing frequency, noroviruses (NoV) GII and HAdV, rotaviruses (RoV), astroviruses (AsV), NoV GI and finally hepatitis E viruses (HEV) were also observed. Nine types of infectious HAdV were identified. HPyV and EV were found in 80% of seawater samples, NoV GII in 60%, HAdV and SaV in 40% and AsV and RoV in 20%. NoV GI and HEV were not detected in seawater. Intact and infectious HAdV-41 were detected in one of the two seawater samples that gave a positive qPCR result. Hepatitis A viruses were never detected in any water types. Analysis of transcriptomic data from giant clams revealed homologues of fucosyltransferases (*FUT* genes) involved in ligand biosynthesis that strongly bind to certain NoV strains, supporting the giant clams ability to bioaccumulate NoV. This was confirmed by the presence of NoV GII in one of the three batches of giant clams placed in a contaminated marine area. Overall, all sample types were positive for at least one type of virus, some of which were infectious and therefore likely to cause public health concerns.

Keywords Noroviruses · Enteric viruses · Adenoviruses · Wastewater · Recreational water · Giant clams · Infectivity · Next-generation sequencing

Introduction

Acute viral gastroenteritis is a significant public health problem and is a primary cause of morbidity worldwide (Glass et al. 2000). Globally, human enteric viruses such as noroviruses (NoV) and rotaviruses (RoV) are responsible for the majority of cases of viral gastroenteritis. They are transmitted by the faecal-oral route, either through person-to-person transmission or by indirect transmission such as by consumption of contaminated food or water. Recreational, irrigation or shellfish growing waters can become contaminated when for instance municipal wastewater containing high levels of enteric viruses is not efficiently treated and enters the environment (Bosch 2007). If these waters contain infectious viruses, this can lead to population health risks (Haile et al. 1999; Le Guyader and Atmar 2007). It is therefore essential to have a method that can efficiently

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detect human enteric viruses in environmental waters, and also determine their infectivity.

Real-time quantitative PCR (qPCR) is the most sensitive and specific method currently used but is unable to discriminate between infectious and non-infectious viral particles (Richards 1999). Other methods are needed to detect infectious viruses in the environment. These include cell culture for culturable viruses and/or alternative molecular methods such as propidium monoazide (PMA) coupled with qPCR, integrated cell culture-qPCR (ICC-qPCR), immunocapture-qPCR and other methods aiming to evaluate the integrity of viral particles (Nuanualsuwan and Cliver 2002; Sano et al. 2010; Ogorzaly et al. 2013a, b; Fongaro et al. 2016). These methods would be particularly useful for viruses that fail to grow efficiently in cell culture. For instance, the conventional cell culture assay for human enteric adenoviruses (HAdV-40 and HAdV-41), considered as the second leading cause of childhood gastroenteritis after RoV (Mena and Gerba 2009), is a rather cumbersome way to quantify infectious virions because they grow slowly. To overcome this issue, a modified ICC-qPCR using 293A cells has been published which leads to a detection of the infectious enteric types within 2 days (Ogorzaly et al. 2013b). Another molecular-based method combining immunocapture and qPCR has been designed (Ogorzaly et al. 2013a) to simultaneously detect the HAdV capsid proteins and the viral genome, two components of intact and potentially infectious viruses.

French Polynesia is a French overseas territory located in the South Pacific. The capital Papeete, with a population of about 26,200 inhabitants in 2012 (ISPF 2012), is located on Tahiti, the most populated island of the country. French Polynesia attracts many tourists with about 169,000 having visited the country in 2012 (ISPF 2012). Wastewater treatment in the country relies mostly on the base of independent sewage management systems consisting either of individual wastewater treatment systems (i.e. a septic tank treatment system for one dwelling) or independent wastewater treatment plants (WWTP), which collect the wastewater from a group of dwellings or buildings with no sewerage system. In 2012, only two towns in Tahiti (Papeete and Punaauia) had public WWTP (CHSP 2013). Public WWTP are responsible for the treatment of wastewater collected through a sewerage system from all or part of one or several municipalities. In both cases, the treated wastewaters are released into the environment either directly into the lagoon or through a fresh water stream which then flows towards the lagoon. Environmental waters close to independent and public WWTP outlets are regularly analysed by the French Polynesian authorities (CHSP) for the presence of faecal bacterial indicators but no detection of enteric viruses in these waters has ever been performed.

In French Polynesia, giant clams (*Tridacna maxima*), mainly found in the shallow waters of the Indian and Pacific oceans, are regularly harvested by the Polynesian population for consumption (Jones et al. 1986). Other bivalves such as oysters and mussels are known to bioaccumulate and retain infectious NoV and are often linked to NoV outbreaks (Le Guyader et al. 2006, 2008; EFSA Panel on Biological Hazards 2011). This is due in part to the presence of fucosyltransferases (*FUT* genes) in these shellfish that are involved in the biosynthesis of histo-blood group antigens (HBGAs), complex carbohydrate ligands that strongly bind to certain NoV strains and are therefore involved in the selective bioaccumulation of NoV (Tian et al. 2007; Maalouf et al. 2010, 2011).

The first aim of this study was to test wastewater samples, collected from different WWTP and receiving environmental waters in the urban area of Papeete, for NoV GI and GII, astroviruses (AsV), sapoviruses (SaV), enteroviruses (EV), hepatitis A viruses (HAV), hepatitis E viruses (HEV), RoV, the human polyomaviruses (HPyV) JC and BK and for HAdV using qPCR assays. The second aim was to determine the presence of infectious viruses in the same samples. For this purpose, HAdV were selected as viral models. Their detection and infectious status are nowadays considered as a clear index of the virological quality of water and food samples (Silva et al. 2011; Wyn-Jones et al. 2011; Wyer et al. 2012). This analysis was carried out using both ICC-qPCR and immunocapture-qPCR methods. Additionally, the diversity of HAdV types was specifically studied using a next-generation sequencing (NGS) approach. Finally, the capability of giant clams to selectively bioaccumulate NoV particles was investigated from both a practical and a theoretical point of view. Giant clams exposed to potentially virus contaminated seawater areas were tested for NoV, and an analysis of the transcriptome of giant clams to identify *FUT* gene homologues was performed.

Materials and Methods

Wastewater and Seawater Sampling

Six 500-mL untreated wastewater samples were collected from four independent WWTP (sites 1, 2, 5 and 6) and two public WWTP (sites 3 and 4) on Tahiti, French Polynesia (Fig. 1; Table 1). Samples were collected at the entrance of the WWTP, before any treatment. More details on the WWTP are shown in Table 1.

Five 10-L seawater samples were collected from three sites where contamination of seawater through wastewater was probable (Sites 7–9) (Fig. 1; Table 1). All samples were stored at 4 °C until analysis.

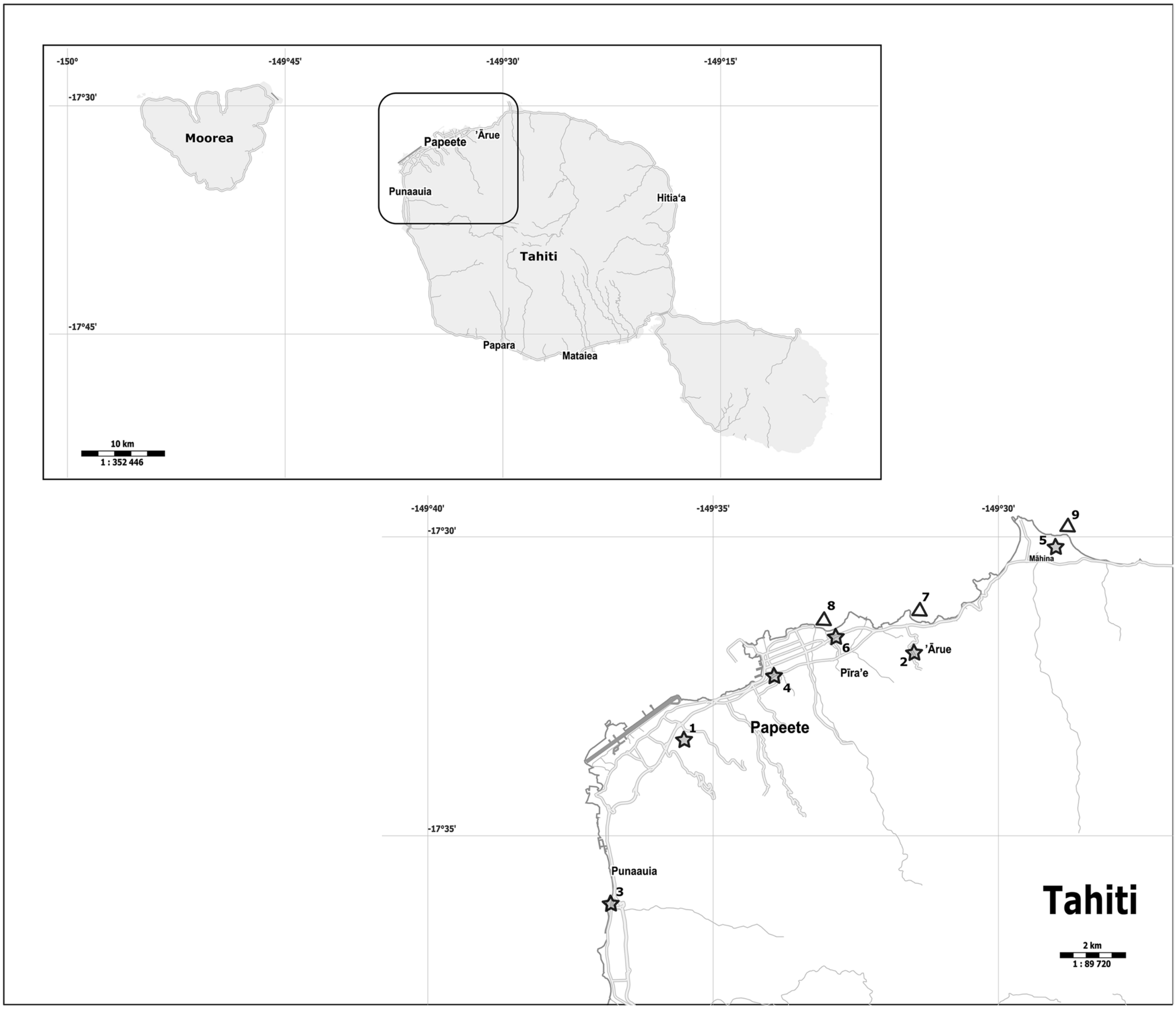

Fig. 1 Sampling locations for wastewater and seawater samples from the urban area of Papeete. The stars and triangles represent the locations of the sampling sites for wastewater and seawater samples, respectively. Numbers represent the sampling site numbers

Wastewater and Seawater Samples Concentration

Methods described by Hewitt et al. (2011) and Hill et al. (2005) were used for the concentration of untreated wastewater and seawater samples, respectively. Briefly, a flocculation with polyethylene glycol (PEG) (Sigma–Aldrich, St. Louis, MO, US) and centrifugation was used to concentrate 500 mL of untreated wastewater samples down to 7 mL. Ultrafiltration followed by a PEG flocculation and centrifugation was used to concentrate 10 L of seawater samples down to 7 mL. For both methods, pellets were re-suspended in phosphate-buffered saline (PBS). The concentrates were stored at − 80 °C until further analysis.

Analysis of NoV in Giant Clams

Study Design

Nine live giant clams were purchased (Tahiti Marine Aquaculture Society). One batch of three individuals was tested at time 0 (T_0). The other individuals were immerged in a cage in seawater, in an area where faecal contamination was regularly observed (Table 1—site 8) through bacterial analyses (CHSP 2016). One batch of three individuals was collected after 48 h of immersion (T_{48h}). The last batch was collected after 1 week of immersion (T_{1week}).

Table 1 Location and details of raw wastewater (a) and seawater (b) sampling sites

(a)

Site	Name	WWTP type	WWTP capacity (m3/day)	Treatment	UV treatment	Chlorination
1	Puurai	Independent	1200	Biological	No	No
2	Erima	Independent	845	Biological	Yes	No
3	Punaauia	Public	14,000	Physico-chemical	No	No
4	Papeete	Public	624	Biological	Yes[a]	Yes[a]
5	Fareroi	Independent	336	Biological	No	No
6	Taaone	Independent	470	Biological	No	Yes

(b)

Site	Name	Number of samples collected
7	Tombeau du roi	1
8	Embouchure Hamuta	2 (collected on different days)
9	Hitimahana	2 (collected on different days)

[a] Equipment was not functioning at the time of collection

The batch $T_{1\text{week}}$ was reduced to one individual as the other two were eaten by predators during the immersion period.

Stomach and Digestive Tissue (SDT) Processing

Each giant clam was shucked, dissected and the stomach and digestive tissues (SDT) retrieved. The shellfish SDT were frozen at − 20 °C until processing. Shellfish SDT was processed using the proteinase K digestion method, as described in the EU CEN method (Jothikumar et al. 2005; Lees 2010). Briefly, an equal volume of 0.2 mg/mL (30 U/mg) proteinase K solution (Sigma-Aldrich, St. Louis, MO, US) was added to each batch of shellfish SDT and pH was adjusted to 8.0–8.3. Samples were incubated for 1 h at 37 °C (with shaking) and then for 15 min at 60 °C. After a centrifugation at 10,000×*g* for 10 min, the supernatants were collected and stored for less than 24 h at 4 °C until viral nucleic acid extraction.

Viral Nucleic Acid Extraction

Viral nucleic acids from 200 µL of the water concentrates or 500 µL of the giant clams concentrates were extracted using the High Pure Viral Nucleic Acid Kit (Roche Molecular Biochemicals Ltd, Mannheim, Germany) following manufacturer's instructions. Nucleic acid extracts were stored at − 80 °C until required.

Reverse Transcription (RT)-qPCR

Wastewater and seawater samples were tested for NoV GI and GII, AsV, SaV, EV, HAV, HEV, RoV, HAdV and HPyV using either reverse transcription qPCR (RT-qPCR) or qPCR assays. Giant clams samples were tested for NoV GI and GII.

Complementary DNA (cDNA) was generated from 2.5 µL viral RNA (NoV GI, NoV GII, AsV, SaV, EV, HAV, HEV and RoV). The RT mix contained 0.1 µM of specific reverse primers for the tested virus (except for EV: 0.25 µM) (Table 2), 50 U Superscript™ III reverse transcriptase (Invitrogen, Carlsbad, CA, US), 5 U RNase inhibitor (RNaseOUT™, Invitrogen), 10 mM dithiothreitol, 1 mM deoxynucleotide triphosphate and 1 × First Strand RT buffer (Invitrogen). The RT reactions were carried out at 50 °C for 30 min followed by 94 °C for 4 min.

Each 25-µL qPCR assay comprised 5 µL of cDNA for RNA viruses or 2.5 µL of extracted DNA for DNA viruses (HAdV, HPyV), 12.5 µL of 2 × Platinum® Taq Quantitative PCR Supermix-UDG (Invitrogen), 0.2 µM of probe (except for HEV: 0.35 µM and for EV: 0.25 µM) and 0.4 µM of primers (except for EV: 0.6 µM and for RoV: 0.3 µM) (Table 2). An initial polymerase activation was carried out at 95 °C for 5 min (or 3 min for HPyV) followed by qPCR-cycling protocol on an RG 3000 or an RG 6000 (RotorGene™, Corbett Life Science, Sydney, Australia), consisting of denaturation at 95 °C for 15 s (except for RoV: 94 °C for 20 s and for EV: 95 °C for 20 s) and annealing/extension for 1 min at a temperature specific to each virus (Table 2) over 45 cycles.

The analysis of raw data was performed using the RotorGene™ software to calculate the qPCR cycle threshold (Cq) values. At least three (RT)-qPCR replicates were performed per test for seawater and wastewater samples. Two extractions and two RT-qPCR replicates per extract were performed for each giant clam's sample. Quantitation of each virus using mean Cq values from the positive (RT)-qPCR replicates was determined using standard curves previously established from serial dilutions of DNA plasmids quantified by spectrometry and analysed by qPCR. For NoV GI, NoV

Table 2 Annealing/extension temperatures and references used for each (RT)-qPCR assay

Virus	Annealing/extension temperature (°C)	References
Norovirus GI	57	Wolf et al. (2010)
Norovirus GII	56	Kageyama et al. (2003)
Murine norovirus	56	Hewitt et al. (2009)
Armored RNA	56	Greening and Hewitt (2008)
Astrovirus	53	Le Cann et al. (2004)
Enterovirus	60	Donaldson et al. (2002)
Hepatitis A virus	60	Costafreda et al. (2006)
Hepatitis E virus	58	Jothikumar et al. (2006)
Human adenovirus—all species	60	Hernroth et al. (2002)
Human polyomavirus	60	McQuaig et al. (2009)
Rotavirus	60	Pang et al. (2004)
Sapovirus	55	Svraka et al. (2010)

GII and HAdV, the theoretical limit of detection (LOD) was 350 genome copies (GC)/L of wastewater and 17.5 GC/L of seawater. For EV, HAV, AsV, SaV, RoV, HPyV and HEV, the theoretical LOD was 467 GC/L of wastewater and 23 GC/L of seawater. The theoretical limit of quantitation (LOQ) was 1400 GC/L of wastewater and 70 GC/L of seawater. The theoretical LOD and LOQ were 20 and 80 GC/g of shellfish SDT, respectively. Box plots, generated with the R software (R Core Team 2014), were used to represent the median concentrations ($\log_{10}$ GC/L), first quartile and third quartile of positive samples. The whiskers reach the minimum and maximum viral concentrations no more than 1.5 time the interquartile range from the box. Viruses that were not detected or with a concentration below the LOQ were not integrated in the boxplot analysis.

Quality Control

For each nucleic acid extraction and each (RT)-qPCR reaction, anti-contamination procedures were followed and positive and negative controls were added. Murine norovirus (MNV) for the water samples and armored RNA (aRNA) for the shellfish samples were added to each concentrate prior to nucleic acid extraction to control for the extraction efficiency and to detect any potential PCR inhibition. A monitoring of the Cq values of the positive controls and of the MNV and aRNA internal controls was performed to detect any potential inhibition and inconsistency between runs.

HAdV Immunocapture-qPCR

Wastewater and seawater samples were tested for the presence of structurally intact HAdV using an immunocapture-qPCR protocol as previously described (Ogorzaly et al. 2013a). Briefly, 50 µL of concentrates was introduced in wells of a 96-well format polypropylene real-time PCR plate (MicroAmp™ Fast Optical 96-Well Reaction Plate, 0.1 mL, Life Technologies, Thermo Fisher Scientific, Waltham, MA, US) previously coated with 50 µL/well of 5 µg/mL monoclonal antibody 8C4 targeting HAdV before DNase treatment (RNase-Free DNase Set, Qiagen, Hilden, Germany). To remove free viral DNA, 100 µL of DNase solution (containing 6.8 Kunitz units DNase I and 10 µL RDD buffer in DNase RNase free water, Qiagen) was added per well and incubated at room temperature for 15 min. After well washing (five times with PBS-Tween and five times with milliQ water), 50 µL of qPCR reaction mix was added in each well and DNA amplification was carried out directly with primers and probe specific of HAdV. A standard curve was established using HAdV-2 to allow the quantification of viral particles in the reaction volume. The immunocapture-qPCR assay displayed an LOD of 10 viral particles per reaction with a dynamic range from 10^2 (LOQ) to 10^6 viral particles per reaction for quantification.

HAdV ICC-qPCR

The ICC-qPCR assay using 293A cells was carried out as previously described (Ogorzaly et al. 2013b). 1 mL of water concentrate was added to a cell monolayer and incubated for 60 min at 37 °C. Afterwards, the inoculum was removed; the cells were washed three times with 4 mL of culture media and incubated for 48 h at 37 °C in presence of 2% culture media containing 1% antibiotics–antimycotic solution (10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin and 25 µg/mL of amphotericin B; Life Technologies). The concentrations of viral genome inside cells after 1 and 48 h of contact were compared, using the following equation $[\mathrm{Cq}\ (T_{48}) - \mathrm{Cq}(T_0)]/s$, where Cq ($T_0$) is the Cq value obtained from cell culture flasks after 1 h of incubation, Cq (T_{48}) is the Cq value obtained after 48 h of incubation and s is the slope of the standard curve. A sample was determined positive when an increase of genome concentration was observed. Appropriate positive (HAdV-2) and negative controls were added at the different steps of the whole process.

NGS for HAdV Typing

The HAdV ICC-qPCR positive water samples were further characterised by amplicon sequencing using a NGS approach. A hypervariable region of the hexon gene, amplified by the degenerated hex1deg–hex2deg primer pair (Allard et al. 2001) and allowing discrimination of HAdV types, was targeted for this assay. Genome amplification from cell culture lysates, DNA library preparation and sequencing were performed according to a previously described protocol (Ogorzaly et al. 2015). After read generation on the benchtop MiSeq platform (Illumina), data were analysed using the Mothur software (Schloss et al. 2009) following the standard operating procedure (SOP) for MiSeq provided on the Mothur platform (http://www.mothur.org/wiki/MiSeq_SOP) (Kozich et al. 2013). Identification of all HAdV species and types simultaneously present in a unique sample was undertaken by comparison of obtained reads with a reference alignment file containing the official sequences for each approved HAdV type.

Transcriptomic Data Analysis

To find fucosyltransferases (*FUT* genes) orthologs in the giant clams genome, a bidirectional-best blastX hit with a threshold of 10^{-3} was used between the contigs issued from the giant clams transcriptomic data (Dubousquet et al. 2016) and a custom human FUT protein database. This database was composed of 9 FUT proteins issued from GenBank (FUT1: NP_000139.1, FUT2: NP_000502.4, FUT2: NP_001091107.1, FUT3: NP_001091110.1, FUT3: NP_001091109.1, FUT3: NP_001091108.1, FUT3: NP_000140.1, FUT6: NP_001035791.1 and FUT6: NP_000141.1).

Results

Viruses in Wastewater

All six wastewater samples were positive for at least one type of virus. Figure 2 shows box plots with the median concentration and concentration range of viruses detected in the wastewater concentrates. Inhibition of the (RT)-qPCR was not detected in these samples. EV, SaV and HPyV were detected in all wastewater samples. Average concentrations of EV and SaV were 2.3×10^5 and 8.3×10^6 GC/L, respectively. HPyV were present at the highest concentration, with an average concentration of 3.4×10^7 GC/L and concentrations ranging from 1.9×10^6 to 1.4×10^8 GC/L (site 3 and site 4, respectively). The viruses with the second highest average concentration were NoV GII (1.7×10^7 GC/L). NoV GII were detected in 5/6 (83.3%) wastewater samples with concentrations ranging between 1.4×10^4 GC/L (site 4) and 8.4×10^7 GC/L (site 2).

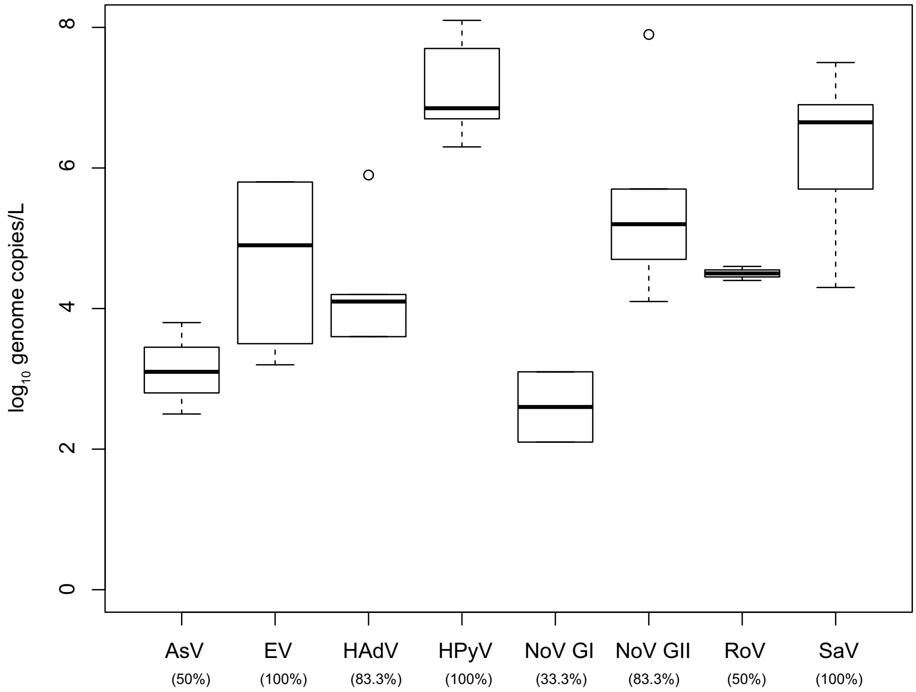

Fig. 2 Concentrations of NoV GI, NoV GII, AsV, EV, HAdV, HPyV, RoV and SaV in wastewater samples from WWTP in the urban area of Papeete. The box plots represent the median concentrations (log$_{10}$ GC/L), first quartile and third quartile of positive samples. The whiskers reach the minimum and maximum viral concentrations no more than 1.5 time the interquartile range from the box. The numbers in parentheses represent the percentage of positive samples for each virus ($n = 6$)

RoV and AsV were detected in half the wastewater samples (50%), in sites 1, 4 and 6 for RoV and in sites 1, 2 and 3 for AsV, at an average concentration of 3.3×10^4 and 2.6×10^3 GC/L, respectively. NoV GI had the lowest average concentration (7.2×10^2 GC/L) and were only detected in 2 out of 6 (33.3%) wastewater samples, with the lowest concentration (1.2×10^2 GC/L) found in site 6 and the highest concentration (1.3×10^3 GC/L) in site 3 (Fig. 2). The least prevalent viruses in wastewater samples were HEV, found only in site 4 (16.7%) and with a concentration lower than the limit of quantitation. HAV were not detected in the analysed wastewater samples.

HAdV were detected in 5/6 (83.3%) of wastewater samples. Concentrations ranged from 3.6×10^3 to 8.2×10^5 GC/L with an average concentration of 1.7×10^5 GC/L (Fig. 2). Using the immunocapture-qPCR assay, all wastewater samples that gave a positive qPCR result for HAdV were under the limit of detection for the presence of structurally intact HAdV particles. Using the ICC-qPCR assay, infectious HAdV were detected in 80% (4/5) of samples (sites 1, 2, 5 and 6) that gave a positive qPCR result for HAdV. Altogether, nine different types of infectious HAdV were identified in the four positive wastewater samples i.e. HAdV-1, HAdV-2, HAdV-3, HAdV-6, HAdV-12, HAdV-29, HAdV-31, HAdV-40 and HAdV-41. Samples from sampling sites 1, 2, 5 and 6 were contaminated with 5, 2, 3 and 3 different HAdV types, respectively (Fig. 3). HAdV-41 were present in the four positive samples and were often the most abundant.

Viruses in Seawater

All five seawater samples tested positive for at least one type of virus. Figure 4 shows box plots with the median concentration and concentration range of viruses detected in the seawater concentrates. Inhibition of the (RT)-qPCR was not detected in these samples, except for the two samples collected in site 8. A 1/10 dilution of these two samples was used and no further inhibition was observed. HPyV and EV were the most prevalent viruses, found in 80% (4/5) of the seawater samples, at an average

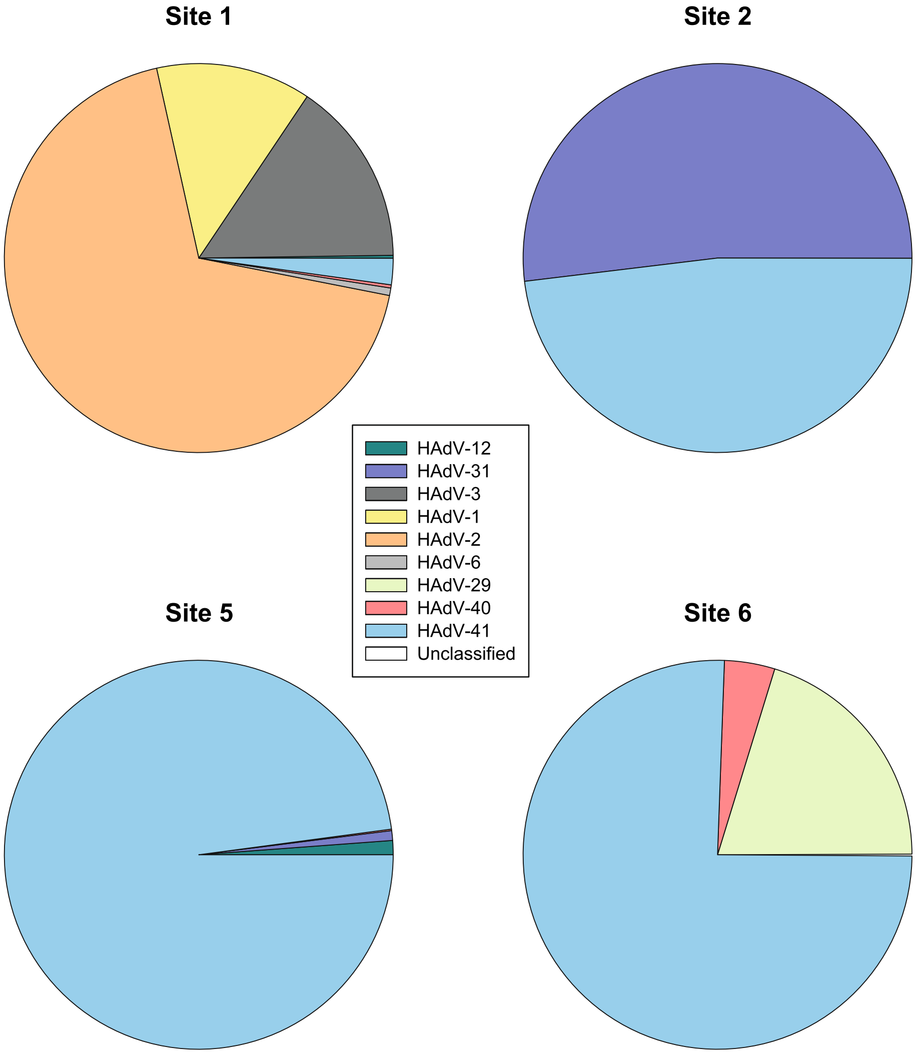

Fig. 3 Diversity of infectious HAdV types recovered from the wastewater samples

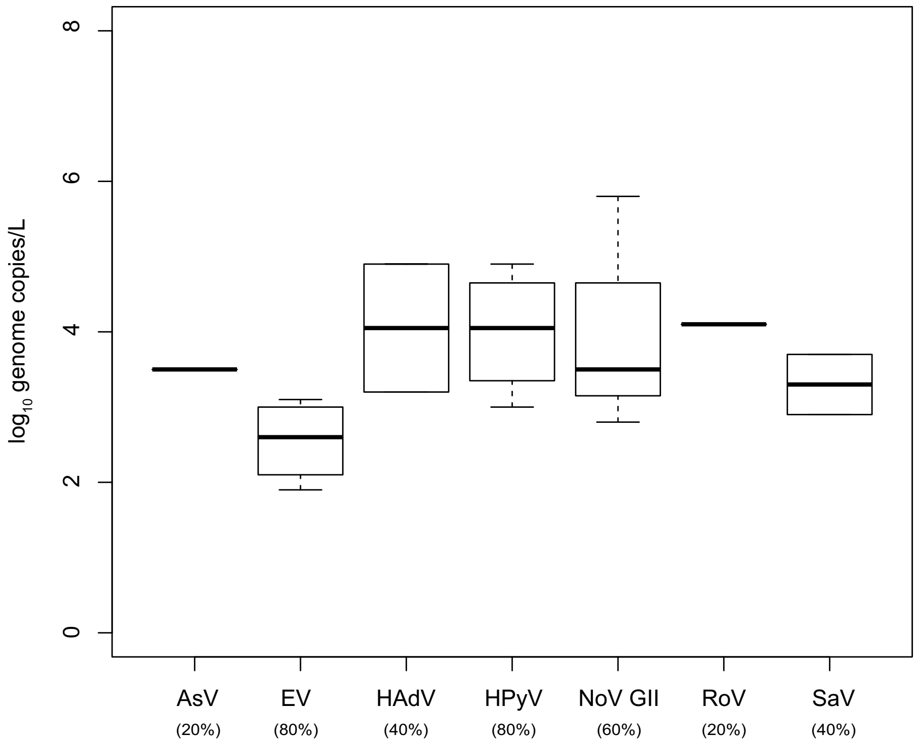

Fig. 4 Concentrations of NoV GII, AsV, EV, HAdV, HPyV, RoV and SaV in seawater samples collected close to WWTP outlets in the urban area of Papeete. The box plots represent the median concentrations (log$_{10}$ GC/L), first quartile and third quartile of positive samples. The whiskers reach the minimum and maximum viral concentrations no more than 1.5 time the interquartile range from the box. The numbers in parentheses represent the percentage of positive samples for each virus ($n=5$)

concentration of 2.6×10^4 and 5.6×10^2 GC/L, respectively (Fig. 4). HPyV were not detected in site 7 and EV were not detected in one out of the two samples collected in site 9. NoV GI were not detected in any of the seawater samples but NoV GII were detected in 60% (3/5; all sites except site 7) of samples with an average concentration of 1.9×10^5 GC/L (Fig. 4). The lowest (6.8×10^2 GC/L) and highest (5.7×10^5 GC/L) concentrations of NoV GII were both detected in site 8. SaV and HAdV were detected in 2 out of 5 (40%) tested seawater samples. SaV concentrations ranged from 8.5×10^2 GC/L (site 9) to 5.5×10^3 GC/L (site 8) with an average concentration of 3.2×10^3 GC/L (Fig. 4).

AsV and RoV were only detected in 1 out of 5 (20%) seawater samples with concentrations of 3.3×10^3 GC/L (site 8) and 1.3×10^4 GC/L (site 8), respectively (Fig. 4). HEV and HAV were not detected in the seawater samples.

HAdV were found in two seawater samples (site 9) at concentrations ranging from 1.5×10^3 to 8.5×10^4 GC/L and with an average concentration of 4.3×10^4 GC/L (Fig. 4). Using the immunocapture-qPCR assay, one (site 9) of the two seawater samples that gave a positive HAdV qPCR result was declared positive, with a Cq value close to the LOQ of this assay (LOQ: 1.5×10^2 viral particles/reaction). The HAdV concentration was estimated at 6.0×10^4 structurally intact viral particles/L of seawater (site 9). With the ICC-qPCR assay, infectious HAdV were detected in the same seawater sample (site 9). Typing assay showed that the infectious HAdV present in this sample were predominantly HAdV-41 (99.9% of assigned contigs). Traces of HAdV-40 (0.1% of assigned contigs) were also highlighted.

Analyses of Giant Clams

Transcriptomic Data

The bidirectional-best blastX hit performed between the giant clams global transcriptome and the sequences of the human FUT proteins, disclosed the presence of at least 4 *FUT* homologues in giant clams genome (*FUT1, FUT2, FUT3* and *FUT6*; Table 3).

NoV in Giant Clams

Batches T_0 and T_{48h} showed PCR inhibition. After 1/10 dilution of the RNA, this inhibitory effect was no longer observed. Out of the three batches of giant clams tested for NoV, one batch (T_{1week}) was positive for NoV GII. All batches were negative for NoV GI.

Discussion

Available data on the circulation of enteric viruses in French Polynesia are limited, with no data on the presence of these enteric viruses in wastewater and environmental waters. In this study, wastewater samples from Tahiti were mostly collected from independent WWTP, which are a simpler

Table 3 Hits of blastX results between giant clams' coding DNA sequences and human fucosyltransferase proteins

Contig query	FUT proteins—accession number	% Identity	Coverage (aa)	*E*-value	Bitscore
cds.comp23434_c0_seq1|m.15001	FUT1 NP_000139	28.62	283	2.00E−24	92.4
	FUT2 NP_001091107	25	300	1.00E−21	84
	FUT2 NP_000502	25	300	1.00E−21	84
cds.comp31438_c0_seq5|m.20090	FUT1 NP_000139	33.33	51	3.00E−04	28.1
cds.comp36181_c0_seq1|m.23096	FUT1 NP_000139	23.74	278	3.00E−23	88.2
	FUT2 NP_001091107	23.36	304	2.00E−22	86.3
	FUT2 NP_000502	23.36	304	2.00E−22	86.3
cds.comp36838_c0_seq1|m.23349	FUT6 NP_000141	36.86	255	2.00E−43	147
	FUT6 NP_001035791	36.86	255	2.00E−43	147
	FUT3 NP_000140	36.9	252	1.00E−41	142
	FUT3 NP_001091108	36.9	252	1.00E−41	142
	FUT3 NP_001091109	36.9	252	1.00E−41	142
	FUT3 NP_001091110	36.9	252	1.00E−41	142
cds.comp36838_c0_seq2|m.23351	FUT6 NP_000141	37.19	242	1.00E−39	135
	FUT6 NP_001035791	37.19	242	1.00E−39	135
	FUT3 NP_000140	37.24	239	8.00E−38	130
	FUT3 NP_001091108	37.24	239	8.00E−38	130
	FUT3 NP_001091109	37.24	239	8.00E−38	130
	FUT3 NP_001091110	37.24	239	8.00E−38	130
cds.comp39298_c0_seq4|m.24567	FUT2 NP_001091107	24	50	1.00E−04	29.3
	FUT2 NP_000502	24	50	1.00E−04	29.3

solution for wastewater treatment than public WWTP, but often lack the required maintenance (CHSP 2013). Effluents from these WWTP flow directly into the lagoon. Regular bacterial analysis carried out close to the WWTP outlets show frequent contamination of the seawater, as determined by elevated *E. coli* and enterococcus levels (CHSP 2016). Faecal pollution in these areas was demonstrated by our study, showing the presence of several enteric viruses in high concentrations in seawater samples. A majority of the tested viruses were found in both wastewater and seawater samples using molecular biology tools. No (RT)-qPCR inhibition was observed for wastewater samples, contrary to seawater samples (2/5, 40%). This was probably due to a different concentration procedure for wastewater and seawater samples. In case of (RT)-qPCR inhibition, a ten-fold dilution was performed before virus quantification.

Some viruses like AsV and RoV were detected in lower concentrations compared to other studies (Le Cann et al. 2004; Aw and Gin 2010; He et al. 2011; Kaas et al. 2016). The average concentration of AsV in wastewater was almost three orders of magnitude lower than the average concentration found in New Caledonia (Kaas et al. 2016) and even lower compared to other studies where AsV concentrations were around 10^7 GC/L in wastewater samples (Le Cann et al. 2004; Aw and Gin 2010). RoV were less prevalent and their average concentration in wastewater was one order of magnitude lower than in studies conducted in New Caledonia (Kaas et al. 2016) and in China (He et al. 2011) where RoV concentrations in wastewater reached 10^5 GC/L. The concentration of RoV detected in the seawater samples was comparable to the ones detected in New Caledonian seawater (Kaas et al. 2016). The concentrations of SaV and EV found in wastewater were similar to others studies conducted in New Caledonia (Kaas et al. 2016) and Japan (Katayama et al. 2008). Their concentrations in seawater were also consistent with levels found in New Caledonian seawater (Kaas et al. 2016) where EV and SaV had average concentrations of 2×10^2 and 1.8×10^3 GC/L, respectively. In 2010, higher seawater concentrations of EV (close to 10^8 GC/L) were reported in China (Zhang et al. 2010).

NoV were detected in both wastewater and seawater samples. NoV GII were found in more wastewater samples than NoV GI and with concentrations more than four orders of magnitude higher. In a study on the concentration and diversity of NoV in Luxembourg wastewaters, Skraber et al. (2011) showed a 30-fold higher concentration of GII than GI. Of the enteric viruses tested, NoV GII were detected in the highest concentrations in the seawater samples. Their incidence (60%, $n = 5$) was consistent with other studies (Tong et al. 2011; Wyn-Jones et al. 2011; Kaas et al. 2016) but the concentration of NoV reported in these studies was lower (around 10^3 GC/L). In a study on the persistence of NoV, Ngazoa et al. (2008) showed that NoV genome can persist for long periods in some water types. This could explain the incidence and concentrations of NoV GII detected in our study. The non-detection of NoV GI in seawater may be because their incidence and concentration in the wastewater flowing into the lagoon were low at the time of the study.

The frequent detection and high concentration of HPyV in this study supports the use of HPyV as potential indicators for the presence of human sewage (Bofill-Mas et al. 2000; Hewitt et al. 2013). Besides HPyV, HAdV are also commonly used as indicators of human faecal pollution in water (Wyer et al. 2012). In our study, HAdV were detected in wastewater with an average concentration similar to the one detected in New Caledonia (Kaas et al. 2016) or Luxembourg (Ogorzaly et al. 2015), but the highest concentration detected was lower than HAdV concentrations in wastewater reported by others (Bofill-Mas et al. 2006; Hewitt et al. 2011) that reached 10^8 GC/L. Their concentration in the seawater samples was almost two orders of magnitude higher than detected in New Caledonian seawater (Kaas et al. 2016). The HAdV diversity study conducted by NGS, using an amplicon approach, showed that nine different types were identified in wastewater samples, while only HAdV-41 was detected in receiving seawater. In wastewater, HAdV-41 was the most abundant and the most frequently observed type followed by HAdV-2 and -31. The others types encountered were HAdV-1, -3, -6, -12, -29 and -40. Most of the types highlighted here were also observed in wastewater in a previous study (Laconelli et al. 2017). A similar sequencing approach was used in a large-scale molecular investigation conducted in Italy with a year-long monitoring of 22 WWTP (Laconelli et al. 2017). The authors identified 19 different HAdV types belonging to HAdV species A to F. HAdV can be responsible for a myriad of clinical illnesses including respiratory, ocular and enteric infections, and encephalitis (Lion 2014). Among them detected in our investigation, types 40 and 41, as unique members of species F, are associated with gastroenteritis and are the most common cause of severe gastroenteritis in children after RoV. Types belonging to the HAdV species A (HAdV-31 and HAdV-12) are significant pathogens in immunocompromised patients in the transplant setting and can be responsible for diarrhea (Jalal et al. 2005; Matsushima et al. 2011). Finally, HAdV types from species B (HAdV-3) and C (HAdV-1, -2, -6) are commonly related to respiratory illnesses. Regardless of the pathology and the HAdV types responsible for the infection, all of them are shed in stools of infected people and then discharged in wastewater treatment plants or directly into surface waters (Mena and Gerba 2009). The NGS results gathered until now clearly show that a large variety of HAdV types can circulate in waters. HAdV-40 and -41 are probably some of the most abundant, but the significance of the others types should not be underestimated in the framework of an environmental risk assessment study.

Of the tested viruses, HAV was the only one to be undetected in either the wastewater or the seawater samples. A study on wastewaters conducted in New Caledonia also revealed the absence of HAV, despite the fact that HAV was circulating within the population as demonstrated by the presence of anti-HAV IgM antibodies in patient samples (Kaas et al. 2016). As no epidemiological data on HAV are available in French Polynesia, the reason for the non-detection of HAV in the tested wastewaters is unclear. HEV was found in wastewater samples at a concentration lower than the LOQ but was not detected in seawater samples. Although there is evidence for the presence of at least HEV genotype 3 in French Polynesia (Le Guern et al. 2015) and in other South-Pacific Islands including New Caledonia where pigs were identified as one reservoir for HEV (Kaba et al. 2011), there are no data on the prevalence of HEV in French Polynesia nor on identified reservoirs.

Even if only a small percentage of the viruses detected in the seawater samples are infectious, the population could be exposed to a health risk during recreational activities as the infectious dose of most enteric viruses is low (Gerba and Haas 1988; Atmar et al. 2014). Some progress has been made over the past years (Ettayebi et al. 2016), but some enteric viruses such as NoV are not yet easily culturable and their infectious state consequently is not easy to determine. The number of reported viral waterborne disease outbreaks associated with recreational water exposures are increasing (Sinclair et al. 2009). The detection of infectious particles in environmental samples using rapid, sensitive and reliable methods remains therefore essential. Methods like the ones used in this study are able to give an overview of the viruses' infectivity. Using the ICC-qPCR assay, a number of wastewater and seawater samples were positive for the presence of infectious HAdV, demonstrating a potential human health risk during recreational activities. Sample analysis carried out using the immunocapture-qPCR assay could not determine the occurrence of intact HAdV particles in wastewater samples. For the seawater samples, 50% ($n=2$) were positive using the same assay with a viral concentration estimated to 6.0×10^4 viral particles/L. The absence of detection in wastewater samples contrary to seawater samples was certainly due to interfering materials impeding the antibody-antigen association, more than to the sensitivity of the assay. Indeed, a positive detection was obtained here for seawater or previously with freshwater samples (Ogorzaly et al. 2013a, b) that were less contaminated than wastewater samples. This showed that the efficiency of the immunocapture-qPCR is affected by sample matrix and type of water, as previously suggested for immunomagnetic separation (Hamza et al. 2011). The interaction of other particles with antibodies or enzymatic reaction should not be neglected. However, the immunocapture-qPCR approach has the advantages to be rapid and relevant for non-culturable viruses. Even if the ability to discriminate between infectious and non-infectious viruses is not fully guaranteed, a selective detection of intact viral particles is performed, rather than naked viral genomes.

The contamination of seawater by potentially infectious viral particles does not only result in an increased health risk during recreational activities, but the collection and consumption of shellfish in these areas could also lead to health problems. Bivalve shellfish are well known as vectors for human pathogens and outbreaks of acute gastroenteritis caused by NoV have been linked to their consumption following contamination of growing waters with human fecal material (Le Guyader et al. 2008; EFSA Panel on Biological Hazards 2011; Greening et al. 2012). A comparison of the transcriptome of giant clams and the sequence of the human *FUT* genes in this study showed that giant clams could potentially express HBGAs, as for mussels and oysters, and therefore would have the ability to bioaccumulate NoV. While our field study showed that giant clams can be contaminated by NoV, we could not conclude that giant clams can selectively bioaccumulate NoV strains. The identification of fucosyltransferases in giant clams are a strong indication that such selective bioaccumulation is possible. Specific interactions as well as strong non-specific interactions both play an important role in the mechanism of bioaccumulation of NoV in bivalves. Further work would be required to assess if these interactions are involved in the binding of different NoV strains to giant clams tissues. Several approaches are available to understand the binding patterns including (i) bioaccumulation studies followed either by viral detection by immuno-histochemistry or by qPCR (Maalouf et al. 2010, 2011); (ii) ELISA studies using monoclonal antibodies (Tian et al. 2006, 2007, 2008), (iii) binding-based RT-qPCR assays (Langlet et al. 2015). Beyond NoV, the bioaccumulation of HEV in bivalve shellfish was previously demonstrated in some European studies (Diez-Valcarce et al. 2012; Grodzki et al. 2012; O'Hara et al. 2018), and the consumption of shellfish has been also reported as a possible source of contamination of HEV in French Polynesia (Le Guern et al. 2015). In this latter, the consumption of giant clams was suspected to be responsible for a case of autochthonous hepatitis E. Our results did not reveal the presence of HEV in the seawater samples and therefore HEV was not tested in giant clams. Further work should be carried out to study the prevalence of HEV in the population in French Polynesia but also to identify potential reservoirs of HEV.

Overall, data gathered during this study provide for the first time insights on the presence of enteric viruses in the French Polynesian environment. Different types of enteric viruses including a majority of NoV GII, HPyV, HAdV, EV and SaV, have been detected at high concentrations in both sewage and receiving seawater. The HAdV, used as index viruses, showed to be infectious. In addition, the presence of viral RNA in giant clams growing in contaminated areas was also demonstrated, and confirms the potential of these shellfish to become contaminated with NoV, like other bivalves such as oysters and mussels. The fecal pollution highlighted here can be responsible for public health concerns for local populations as well as for tourists through contact with contaminated water during recreational, fishing or bathing activities, or through the consumption of shellfish.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Approval The manuscript does not contain clinical studies or patient data.

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