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ORIGINAL PAPER

Surveillance of Enteric Viruses and Thermotolerant Coliforms in Surface Water and Bivalves from a Mangrove Estuary in Southeastern Brazil

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Abstract

This study was conducted to evaluate the microbiological quality of a mangrove estuary in the Vitória Bay region, Espírito Santo, Brazil. We analyzed the presence and concentration of enteric viruses and thermotolerant coliforms in water, mussels (*Mytella charruana* and *Mytella guyanensis*), and oysters (*Crassostrea rhizophorae*), collected over a 13-month period. Human adenovirus, rotavirus A (RVA), and norovirus genogroup II were analyzed by quantitative PCR. The highest viral load was found in RVA-positive samples with a concentration of 3.0×10^4 genome copies (GC) L^{-1} in water samples and 1.3×10^5 GC g^{-1} in bivalves. RVA was the most prevalent virus in all matrices. Thermotolerant coliforms were quantified as colony-forming units (CFU) by the membrane filtration method. The concentration of these bacteria in water was in accordance with the Brazilian standard for recreational waters (< 250 CFU 100 mL^{-1}) during most of the monitoring period (12 out of 13 months). However, thermotolerant coliform concentrations of 3.0, 3.1, and 2.6 log CFU 100 g^{-1} were detected in *M. charruana*, *M. guyanensis*, and *C. rhizophorae*, respectively. The presence of human-specific viruses in water and bivalves reflects the strong anthropogenic impact on the mangrove and serves as an early warning of waterborne and foodborne disease outbreaks resulting from the consumption of shellfish and the practice of water recreational activities in the region.

Keywords Enteric virus · Thermotolerant coliform · Bivalve mollusk · Mangrove · qPCR

Introduction

Mangroves are ecosystems of great environmental importance because they provide ideal shelter conditions for marine life, serving as breeding and nursery areas for several species that inhabit the oceans (Kathiresan and Bingham 2001). In addition, mangroves have major social and economic importance for local communities that depend on mangrove resources for food and income. The contamination of marine resources is a public health threat, particularly regarding filtering organisms, such as bivalve mollusks, which concentrate contaminants and pathogenic microorganisms from the water (Dore et al. 2010). Foodborne disease outbreaks associated with shellfish consumption have been reported in many parts of the world. Most cases are related to the consumption of oysters, clams, mussels, or cockles (Morse et al. 1986; Potasman et al. 2002; Wall et al. 2011; Alfano-Sobsey et al. 2012; Bellou et al. 2013). Norovirus (NoV), hepatitis A virus (HAV), and rotavirus group A (RVA) are the most frequently reported viruses in foodborne acute gastroenteritis outbreaks (Le Guyader et al. 2008; Guillois-Bécel et al. 2009; Amaral et al. 2015).

Degradation of aquatic environments used for food collection and recreational activities is a common problem worldwide, especially in countries where sanitation is poor or lacking. In Vitória, capital of the state of Espírito Santo, Southeastern Brazil, 2000 ha of mangrove forests surrounding Vitória Bay has been severely impacted in the last two

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decades (Jesus et al. 2004; Souza et al. 2012; Barbirato et al. 2016; Zamprogno et al. 2016). The affected area is close to Ilha das Caieiras, a neighborhood inhabited by a low-income fishing community that survives from the trade and consumption of seafood extracted from the mangrove, such as fish, crabs, shrimps, mussels, and oysters. Restaurants in Ilha das Caieiras attract a significant number of tourists to the area.

Our study aimed to quantify pathogenic enteric viruses (HAdV, RVA, and NoV) and thermotolerant coliforms, which serve as indicators of fecal contamination, in water and bivalves mollusks collected from Vitória Bay to evaluate the impact of anthropogenic activities on the region and thus provide support for pollution management plans and the rehabilitation of this water ecosystem.

Materials and Methods

Collection of Water and Bivalve Samples

Water and bivalve samples were collected monthly from January 2011 to January 2012 at two sites of the Vitória Bay region: site 1 (S1), 20°15′64″S 40°19′960″W, located near the mouth of the Santa Maria da Vitória River, and site 2 (S2), 20°16′262″S 40°20′632″W, located in a preserved area of the mangrove forest, in a small island about 1 km south from S1 and 1 km north from the Bubu River (Fig. 1). This region is drained by rivers that received untreated sewage discharges from the population around the Vitória Bay.

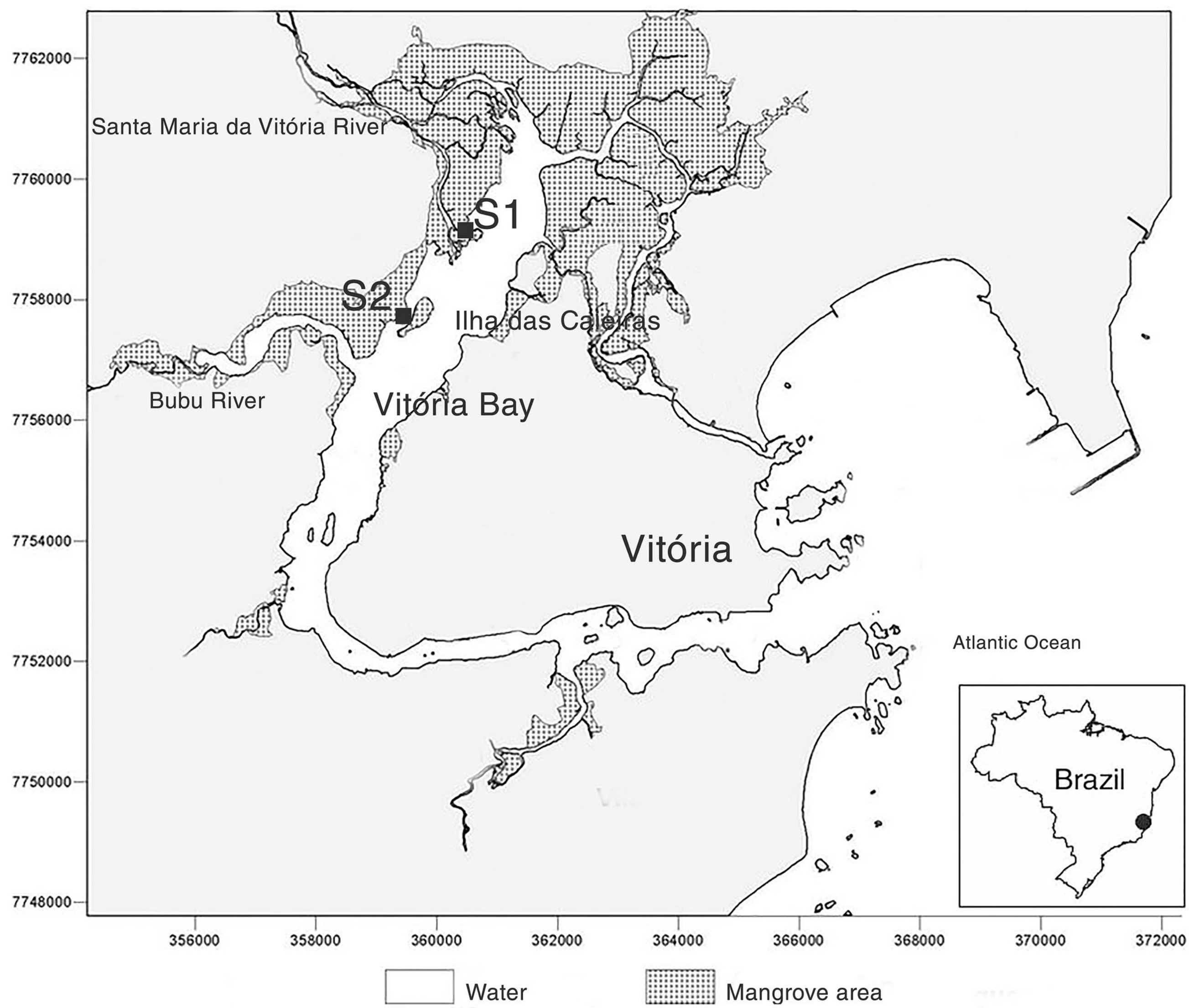

Fig. 1 Location of sampling sites in Vitória Bay: S1, collection of water and Mytella charruana, and S2, collection of water, Mytella guyanensis, and Crassostrea rhizophorae

In total, 26 water samples (13 samples per site) and 30 mussel and oyster samples (13 samples of *M. charruana*, 4 samples of *M. guyanensis,* and 13 samples of *C. rhizophorae*) were collected. *M. guyanensis* samples were collected only in the first four months (January 2011 to April 2011) because there was a marked decrease in the number of individuals of this species during the study period.

Collections were carried out in the morning when the water depth was approximately 0.8 m, as previously calculated using the tide table of the National Oceanographic Data Center. This water level was sufficiently low to allow the visualization and collection of bivalve species, which are covered by the waters of the estuary at higher tides. After collection, samples were refrigerated at 4 °C, transported to the laboratory, and analyzed within 2 h.

Microbiological Analyses

Viral concentration

Viruses were concentrated from water according to the method of Katayama et al. (2002), with modifications. Briefly, 1 L of estuarine water was mixed with 1 L of Tris/Ca^{+2} buffer (0.01 M, pH 7.2). The solution was filtered through a 1.2-µm cellulose ester membrane (Millipore) to remove larger particles, the pH was adjusted to 3.5, and the solution was then filtered through a negatively charged cellulose ester membrane with a pore size of 0.45 µm (Millipore). The membrane was eluted with 12 mL NaOH 10 mM and the eluate was neutralized with 50 µL H_2SO_4 50 mM and 50 µL Tris–EDTA buffer 100×. The eluate was transferred to Amicon Ultra-15 centrifugal filter units (Millipore) and centrifuged at 6700×*g* and 4 °C, resulting in a final volume of 800 µL.

To recover viruses from mussels and oysters, we used the method previously described by Traore et al. (1998), with modifications. Briefly, 10 g of tissue was homogenized with glycine–NaCl buffer (0.1 M and 0.3 M, respectively) (pH 9.5) using a mixer (Walita, Brazil). After centrifugation of the sample (6700×*g* at 4 °C for 30 min), the supernatant was collected and the pH was adjusted to 7.5. Viral particles were precipitated by the addition of a volume of PEG–NaCl (16%, 0.6 M) equal to that of the supernatant. The mixture was stirred for 12 h at 4 °C. After centrifugation (6700×*g* at 4 °C for 30 min), the pellet was resuspended in 0.15 M Na_2HPO_4 buffer (pH 9.0). Another centrifugation step was performed, and a 400 µL aliquot was collected from the supernatant and stored at −20 °C until nucleic acid extraction.

Nucleic Acid Extraction and cDNA Synthesis

Nucleic acids were extracted according to Boom et al. (1990). Reverse transcription was carried out as previously described by Iturriza-Gomara et al. (1999), with modifications. A mixture containing 5 µL of nucleic acid and 1 µL of DMSO was denatured at 97 °C for 7 min and kept on ice for 2 min. Then, the mixture received the addition of a solution containing 10× reaction buffer, 200 µM each dNTP, 2.5 mM $MgCl_2$, random hexanucleotide primers (pdN6™, Amersham Biosciences), and 60 U Superscript II Reverse Transcriptase (Invitrogen). The mixture was incubated at 42 °C for 1 h and denatured at 95 °C for 10 min. cDNA was stored at − 20 °C for subsequent amplification.

Viral Detection and Quantification

Qualitative nested PCR was performed to identify samples positive for the viruses. Positive samples were then quantified by real-time quantitative PCR (qPCR). Qualitative PCR was performed on a PTC-100 v. 7.0 thermal cycler (MJ Research Inc., MA). qPCR was performed on a StepOne™ Real-Time PCR System (Applied Biosystems™, NY, USA). HAdV, RVA, and norovirus GII viral load were determined using standard curves generated from tenfold serially diluted plasmids (10^6 to 10^0 plasmid copies/reaction) containing the target qPCR region for each virus. All samples were analyzed in duplicates. RNAse/DNAse-free water and also a non-template control were included in each run to evaluate possible contamination. The sequences of primers and probes, region of amplification, amplicon size, and references for all PCR assays are available in Online Resource 1 (Table S1).

Bacteriological Analysis

Detection and quantification of thermotolerant coliforms in water and bivalves were carried out using the membrane filtration method, as recommended by APHA (2005). Estuarine water (100 mL) was serially diluted (1:10) before analysis. Mussels and oysters were washed with tap water and opened using a sterile knife for removal of the digestive diverticulum. Then, 25 g of tissue (consisting of approximately six individuals) was homogenized with 1% peptone water using a mixer (Walita, Brazil). Serial dilutions (1:10) were performed according to ISO 16140 (2003). Results are expressed in colony-forming units (CFU) per 100 milliliters (CFU 100 mL^{-1}) of filtered volume for water samples and per 100 grams (CFU 100 g^{-1}) of tissue for bivalve samples.

Physicochemical Analyses and Rainfall Data

Physicochemical analyses were performed on water samples collected from S1 and S2. Conductivity, pH, salinity, turbidity, and dissolved solids were analyzed according to the Standard Methods for the Examination of Water and Wastewater (APHA, AWWA, WEF 2005). Dissolved oxygen concentration was measured according to Winkler (1888). Temperature measurements were taken in the field. Rainfall data for Vitória, ES, were collected by the *Espírito Santo Research, Technical Assistance, and Rural Extension Institute* (INCAPER). Rainfall was evaluated using the accumulated precipitation of the 5 days prior to the day of water collection.

Statistical Analysis

Descriptive statistical analysis was performed on physicochemical results (median, minimum, and maximum values), bacteriological results (geometric mean, median, minimum, and maximum values), and viral results (geometric mean, minimum, and maximum values). The scores of the studied variables were non-normally distributed and thus non-parametric statistics, including Kruskal–Wallis test and Student's t test were used to determine statistical differences among variables. The Spearman coefficient was calculated to investigate correlations between physicochemical parameters, rainfall data, and the presence of viruses in bivalves. The level of significance was set at $p < 0.05$. Analyses were performed using Prism v. 6.1 (GraphPad Software Inc., CA, USA).

Results

Viral Detection

Enteric viruses were monitored in water and bivalve samples collected at two sites in Vitória Bay over a 13-month period. Figure 2 shows the frequency of viral detection in water and bivalves. Of the 26 water samples analyzed, 21 (80.8%) were positive for at least one of the viruses. HAdV, RVA, and NoV were detected in 46% (12/26), 65.4% (17/26), and 27% (7/26) of the water samples, respectively.

Of the 30 bivalve samples, 27 (90%) were virus-positive: 11 (41%) samples were positive for only one virus; 9 (33%) samples were positive for two viruses, mainly RVA and HAdV; and 7 (26%) samples were positive for all viruses. For *M. charruana*, *M. guyanensis*, and *C. rhizophorae* samples, HAdV was detected, respectively, in 61.5% (8/13), 75% (3/4), and 23% (3/13); RVA was detected, respectively, in 69.2% (9/13), 25% (1/4), and 46.2% (6/13); and NoV was detected, respectively, in 54% (7/13), 50% (2/4), and 85% (11/13).

We analyzed the seasonal distribution of viruses in water and bivalves (Fig. 3). Viruses were detected in all matrices throughout the entire year, except for May, when water samples were negative for all viruses. However, in May, NoV GII was detected in *M. charruana* (S1) and *C. rhizophorae* (S2). Table 1 shows the viral load of HAdV, RVA, and NoV GII in water and bivalves.

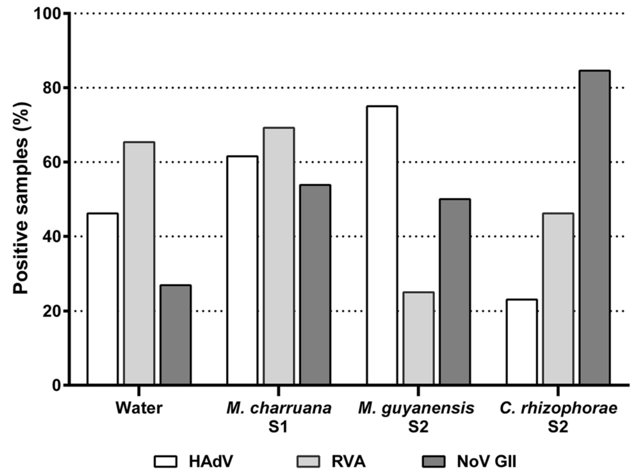

Fig. 2 Frequency of human adenovirus (HAdV), rotavirus A (RVA), and norovirus GII (NoV GII) in water (S1 and S2), mussels (Mytella charruana, S1, and Mytella guyanensis, S2), and oysters (Crassostrea rhizophorae, S2)

Bacterial Analysis

Figure 4 shows the mean concentration of thermotolerant coliforms in water ($n = 26$), *M. charruana* ($n = 13$), *M. guyanensis* ($n = 13$), and *C. rhizophorae* ($n = 4$) samples. The temporal distribution (from January 2011 to January 2012) of thermotolerant coliforms in water and bivalves is presented in Online Resource 2 (Fig. S1). Thermotolerant coliforms were detected in water at concentrations ranging from 1.0×10^1 to 1.4×10^3 CFU 100 mL^{-1} (geometric mean of 1.3×10^2 CFU 100 mL^{-1}). In bivalves, concentrations ranged from 2.0×10^4 to 3.6×10^6 CFU 100 g^{-1} for *M. charruana*, from 2.8×10^4 to 1.2×10^6 CFU 100 g^{-1} for *M. guyanensis*, and from 4.0×10^3 to 2.77×10^5 CFU 100 g^{-1} for *C. rhizophorae*. No statistical difference was observed between the concentration of thermotolerant coliforms in S1 and S2 water samples. The mean concentration of thermotolerant coliforms was higher in bivalves than in water ($p < 0.05$).

Physicochemical Analysis

Table 2 shows the physicochemical properties of water collected in the Vitória Bay region. No significant differences were observed between S1 and S2 samples regarding turbidity ($p = 0.4257$), total dissolved solids ($p = 0.0650$), pH ($p = 0.1745$), temperature ($p = 0.4302$), and dissolved oxygen concentration ($p = 0.4576$).

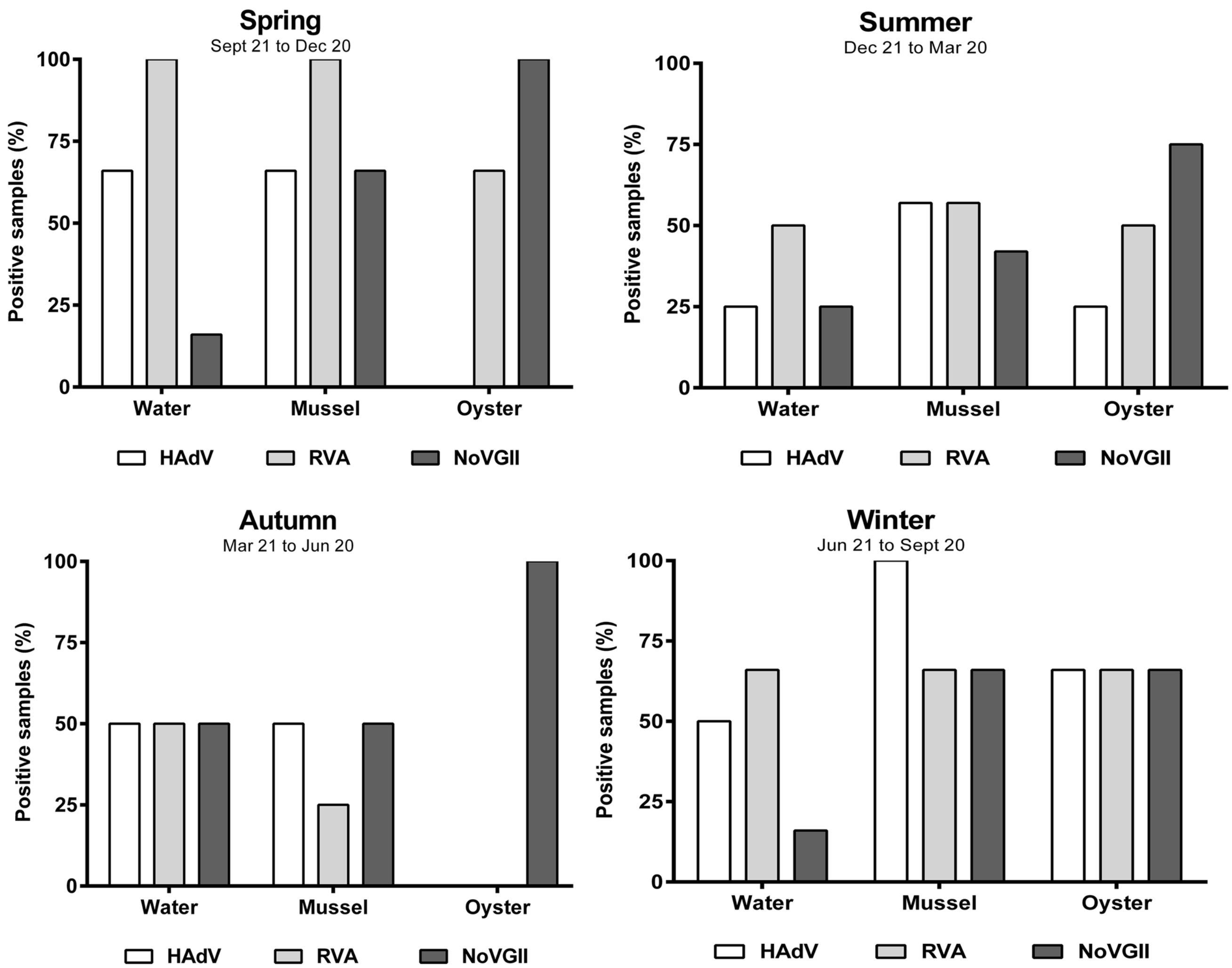

Fig. 3 Seasonal distribution of human adenovirus (HAdV), rotavirus A (RVA), and norovirus GII (NoV GII) in water, mussels (Mytella charruana and Mytella guyanensis), and oysters (Crassostrea rhizophorae)

Table 1 Viral load of human adenovirus (HAdV), rotavirus A (RVA), and norovirus genogroup II (NoV GII) in water and bivalve samples as determined by quantitative PCR

Site	Sample	Number of samples analyzed/GC (geometric mean of positive samples)		
		HAdV	RVA	NoV GII
S1	Water	5 (8.8×10^1)	7 (8.8×10^2)	3 (4.3)
	Mytella charruana	7 (4.5×10^2)	7 (3.4×10^3)	5 (4.0×10^1)
S2	Water	4 (1.1×10^2)	7 (1.1×10^3)	3 (5.4)
	Mytella guyanensis	2 (5.7×10^2)	2 (2.6×10^3)	2 (5.4×10^1)
	Crassostrea rhizophorae	4 (1.2×10^3)	7 (5.3×10^3)	4 (1.9×10^1)

GC Genomic copies per liter (water samples) or per gram (bivalve samples)

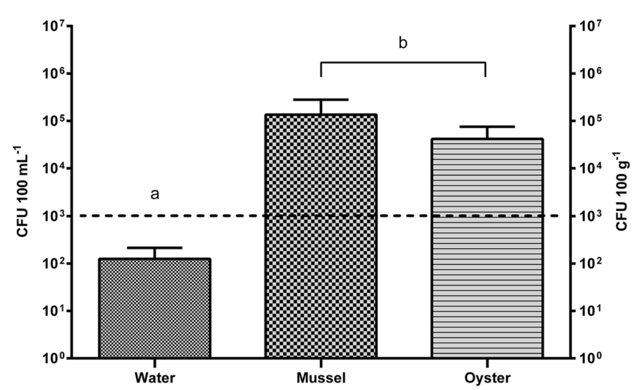

Fig. 4 Mean concentration of thermotolerant coliforms in water, mussels (Mytella charruana and Mytella guyanensis), and oysters (Crassostrea rhizophorae) collected in Vitória Bay. The dotted line indicates the limit of thermotolerant coliforms for recreational water quality established by Brazilian legislation (CONAMA 274/2000). Different letters indicate significant differences by Kruskal–Wallis test ($p < 0.05$)

Table 2 Descriptive statistics of physicochemical water parameters during the 13-month monitoring period in site 1 (S1) and site 2 (S2) of Vitória Bay

Parameter	Site	Median value	Minimum value	Maximum value
Turbidity (NTU)	S1	8.8	2.4	129.9
	S2	8.5	2.7	35.4
Conductivity (mS cm^{-1})	S1	11.5	0.7	38.1
	S2	29.0	2.9	44.9
Total dissolved solids (mg L^{-1})	S1	1.6×10^5	1.9×10^4	1.0×10^6
	S2	4.1×10^5	1.1×10^5	1.2×10^6
Dissolved oxygen (mg L^{-1})	S1	16.0	6.4	16.8
	S2	12.0	8.4	16.8
Salinity (SPU)	S1	11.0	0.0	26.0
	S2	19.0	7.0	27.0
pH	S1	7.3	6.8	8.9
	S2	7.6	6.9	8.7
Temperature (°C)	S1	25.0	22.0	30.0
	S2	25.0	22.2	29.0

Discussion

Detection of HAdV, RVA, and NoV GII, as well as indicator bacteria, throughout the monitoring period in the neighborhood of Ilha das Caieiras, Vitória, Brazil, revealed the poor microbiological quality of water and bivalves collected in the mangrove area. The proximity of both sampling points to the mouths of the Bubu and Santa Maria da Vitoria Rivers, which are possibly contaminated by sewage, could explain these results. We highlight that the region is not designated for commercial harvesting of seafood and its waters are not monitored for microbiological quality, as does occur on shellfish farms. Previous studies showed that shellfish grown in wild areas harbor a greater variety of viruses and have higher co-infection rates than shellfish grown on farms (Romalde et al. 2002; Vilariño et al. 2009). The mangrove, in addition to providing an income source for local fishers, attracts tourists who partake in recreational activities in the slow moving waters. Thus, the presence of more than one type of virus in water and bivalves represents a high risk of exposure to viruses associated with gastrointestinal diseases (Le Guyader et al. 2008; Bellou et al. 2013; Le Mennec et al. 2017).

A high prevalence of HAdV, RVA, and NoV in water and bivalves has been reported by many authors in various geographic areas (Le Guyader et al. 2000; Miagostovich et al. 2008; Keller et al. 2013; Osuolale and Okoh 2015). HAdV, mainly Human adenovirus species F (HAdV-F) types 40 and 41, are commonly associated with acute diarrheal disease (ADD) (Uhnoo et al. 1984; Rezaei et al. 2012). In a recent study (Portes et al. 2016), other HAdV species were also related to acute gastroenteritis. The viruses are released in the feces of infected individuals and can reach the environment by the discharge of sewage into water bodies. HAdV has been detected in sewage effluents, polluted seawater, and shellfish (Girones et al. 1995; Pina et al. 1998; Schlindwein et al. 2010; Osuolale and Okoh 2015). In our study, HAdV was the second most prevalent virus; it was detected in 46% of water samples, 61.5% of *M. charruana* samples, 75% of *M. guyanensis* samples, and 23% of *C. rhizophorae* samples. HAdV was the virus most frequently detected during the winter (June to September).

RVA was prevalent in water and *M. charruana* samples (69.2%, 9/13). The high viral load of RVA detected in water and bivalves indicates that the virus poses a danger to local seafood consumers and tourists. Studies in Brazil reported the environmental dissemination of RVA despite high RVA vaccination coverage since 2006, when the vaccine was included in the national vaccination program (Ferreira et al. 2009; Victoria et al. 2014). Although RVA is one of the main causes of childhood diarrhea worldwide (Parashar et al. 2006; Lanata et al. 2013), interestingly, in adults it has not been linked with gastroenteritis outbreaks related to seafood consumption, neither has HAdV. RVA and HAdV infection in adults generally manifests with mild symptoms or may be asymptomatic. Age-related resistance to severe infection might be due to active immunity, reinforced by repeated infection throughout life (Lees 2000). However, in communities that depend on shellfish as a source of income, such as the Ilha das Caieiras community, shellfish are the main food source for adults and children alike.

NoV GII is the main cause of epidemic gastroenteritis after the consumption of bivalves collected from contaminated areas and many outbreaks have already been reported (Prato et al. 2004; Le Guyader et al. 2009; Smith et al. 2012; Campos and Lees 2014). NoV is highly resistant to environmental degradation in aquatic environments, its persistence is higher during the winter (Campos and Lees 2014; Kauppinen et al. 2017), and its abundance and distribution depend greatly on survival conditions in the receiving water (Seitz et al. 2011). Many studies documented a higher prevalence of NoV GI and NoV GII in the cold seasons (Formiga-Cruz et al. 2002; Lowther et al. 2012; Mounts et al. 2000; Rajko-Nenow et al. 2012), but winter seasonality of NoV GII was not observed in the present study. In a previous study conducted in the same area (Keller et al. 2013), NoV GII was detected in 4.8% of water samples collected from site 2 (S2). In the present study, NoV GII was positive in 38.5% (5/13) of water samples in this sampling location. NoV GII was detected in 50.0% and 85% of *M. guyanensis* and *C. rhizophorae* samples, respectively. Despite the high frequency of NoV GII in mussels and oysters, the viral load was low, ranging from 1.43×10^1 to 9.53×10^1 GC g^{-1}. Similar results were reported for mussels (Le Guyader et al. 2009; Hassard et al. 2017). Unlike enteric bacteria, enteric viruses can initiate infection at very low numbers. For instance, 10–100 virus particles correspond to infectious doses reported for NoV GII and HAdV, respectively (Teunis et al. 2008). Whereas mussels are usually cooked before consumption, oysters are generally eaten raw. Even a low viral load of NoV GII in oysters might pose a potential risk of gastroenteritis (Campos and Lees 2014; Hassard et al. 2017).

We also evaluated the bacteriological quality of water samples. Thermotolerant coliform concentrations were below the limit for recreational water quality, as established by the Brazilian National Environment Council (CONAMA) Resolution no. 274/2000 (Brazil 2000). According to the Resolution, water samples are considered improper if 80% or more of samples obtained for five consecutive weeks contain 1.0×10^3 CFU thermotolerant coliforms 100 mL^{-1} or if the last sample has more than 2.5×10^3 CFU thermotolerant coliforms 100 mL^{-1}. Samples collected in December 2011 contained thermotolerant coliforms at concentrations above the limit considered "proper" but slightly below the limit considered "improper." According to the estuarine water quality standard established by CONAMA (Resolution no. 357/2005, Brazil 2005), the monitored area was considered inadequate for bivalve cultivation, as the limit of thermotolerant coliform concentration was exceeded by up to 30 and 20 times at S1 and S2, respectively, throughout the entire monitoring period. Bacterial concentrations in *C. rhizophorae*, *M. charruana*, and *M guyanensis* were 400, 1000, and 1350 times higher, respectively, than those in water. In the study reported by Keller et al. (2013), the density of bacteria in *M. guyanensis* was 400 times higher than that of the surrounding water. Comparing both studies, our results show that a significant increase in contamination has occurred over the years ($p = 0.0009$).

Physicochemical water parameters were investigated to characterize the estuary in which bivalves were collected and evaluate their influence on the presence of microorganisms in water and bivalves. The physicochemical conditions of a water body may contribute to the persistence of bacteria and viruses in the environment and aquatic organisms. Suspended particles are natural carriers of viruses in water and contribute to virus survival, as they are able to aggregate into larger particles, attach to organic matter, or accumulate in the sediment, thereby protecting viruses from UV damage (Marino et al. 2005; Oliveira et al. 2011). We observed a linear relationship between RVA and total dissolved solids (TDS), between HAdV and turbidity, and between HAdV and TDS. The occurrence of NoV was not correlated with any physicochemical parameter. Phanuwan et al. (2006) showed that *Escherichia coli* and enteric virus concentrations in groundwater and fluvial water increased as water turbidity increased and conductivity and temperature decreased. In our study, pH and temperature did not vary greatly over the monitoring period, and no correlation was observed between these parameters and the presence of viruses in the environment. All viruses were detected in dry and rainy seasons, and there was no statistical correlation between the amount of rain and the concentration of virus particles. Furthermore, there was no significant variation in thermotolerant coliform concentrations and thus no correlation between thermotolerant coliforms and physicochemical water parameters.

This study showed that the pathogenic enteric viruses HAdV, RVA, and NoV GII were detected with high frequency and at high viral load in water, mussels, and oysters collected in the mangrove area of Vitória Bay. During most of the monitoring period, the levels of thermotolerant coliforms in water samples were below the limit for recreational water quality established by Brazilian legislation but above the limit for bivalve farming, demonstrating that the area was not suitable for bivalve cultivation and trade. These findings are important given that oysters are generally consumed raw and are implicated in gastroenteritis outbreaks. Therefore, testing for both fecal indicator bacteria and enteric viruses should be conducted routinely in the Vitória Bay region to ensure the safety of the population during water recreational activities and the safety of fishers and shellfish consumers.

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