Assessment of water and seafood microbiology quality in a mangrove region in Vitória, Brazil

Regina Keller, Juliana F. Justino and Sérvio Túlio Cassini

ABSTRACT

Mangroves are vital part of the local economy for some communities in the region of Vitória, Brazil. Oysters, mussels, and crabs, which are naturally abundant in the mangroves, are harvested and largely consumed in restaurants and by the population. In recent years, unusually high rates of annual gastroenteritis cases have been reported in the region suggesting an association between the consumption of contaminated shellfish and the development of gastrointestinal diseases. The objective of this study was to evaluate water samples and mussels collected in the mangrove region using bacterial indicator *Escherichia coli* and enteric viruses (adenovirus, rotavirus, and norovirus). Our results showed that the region of study is impacted by a continuous discharge of domestic sewage. Although *E. coli* was detected at low densities in water samples, mussels were shown to be 400 times more contaminated throughout the period of the study. Adenovirus and rotavirus genomes were detected by nested-polymerase chain reaction respectively in 76 and 88% of water samples and 100% of mussel samples. Norovirus was found in 4.8% of water samples and was not detected in the mussels. The screening of bivalves for the presence of health-significant enteric viruses can help in the prevention of outbreaks among shellfish consumers and contribute to improvement of the estuarine environment.

Key words | enteric viruses, *Escherichia coli*, mangrove, mussels, water quality

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INTRODUCTION

The contamination of natural bodies of water by pathogenic microorganisms has become a worldwide public health problem, leading to outbreaks of waterborne diseases and a high incidence of morbidity and mortality. Epidemiological evidence suggests that human enteric viruses are the most common etiological agents transmitted by bivalve shellfish, filter-feeding animals that can concentrate pathogenic microorganisms in water contaminated with sewage. The consumption of raw or poorly cooked seafood, such as oysters and mussels, can cause gastrointestinal illness (Croci *et al*. 2000; Pommepuy *et al*. 2006). Enteric pathogens can survive in the environment for weeks to months, either in the water column or adsorbed on particles, and can accumulate in sediments. Among the bivalves, oysters are the predominant vectors of disease, however mussels are also responsible for many outbreaks (Lees 2000).

In Brazil, few studies regarding the bacterial and viral contamination of mangrove areas have been performed. The region of study is a natural nursery that has been repeatedly exploited by local fishermen for several generations. In recent years, fishermen have observed a clear degradation of the environment in terms of the disappearance of mussels and oysters from their usual collection locations. In addition, high rates of annual gastroenteritis cases have been reported in the region, suggesting an association between the consumption or handling of contaminated mussels and the development of gastrointestinal diseases. This study evaluates the sanitation of the mussels and the surrounding water in the mangrove region of Vitória through the bacterial indicator *Escherichia coli* (*E. coli*) and enteric viruses adenovirus (AdV), rotavirus (RV), and norovirus (NV).

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METHODS

The research site is a mangrove area of Vitória Bay, located in Espírito Santo, a state in the southeast region of Brazil. Surrounding water and mussels (*Mytella guyanensis* Mollusca: Bivalvia) were sampled monthly and analyzed for *E. coli*, AdV, RV, and NV. The mussel *Mytella guyanensis* was chosen for this study because of its abundance, wide distribution, and frequent consumption along the Brazilian coast. Moreover, mussels represent an important nutritional resource for low-income populations living in coastal areas of the country.

Between February 2008 and March 2009, 42 1.5 L water samples were collected at three sites (S1, S2, and S3): S1 is located near the river discharge; S2 is on the mangrove banks; S3 is near the pier and a residential area (Figure 1). Eleven mussel samples collected directly from the mangrove roots near S2 were also analyzed. Water samples and mussels were always collected in the morning when the tide was high enough to collect the water samples (±1 m) and the mussel samples (±0.3 m). All samples were refrigerated at 4 °C and transported to the laboratory to be analyzed within a maximum of 2 hours.

Laboratory analysis

Physicochemical analysis

Environmental parameters, such as rainfall (amount of rain accumulated in the 5 days preceding the day of water collection), conductivity, pH, turbidity, and total dissolved solids (TDS) were evaluated according to the *Standard Methods for Water and Wastewater Examination* (American Water Works Association 1995).

Bacterial analysis

The detection and quantification of *E. coli* in water was done using the enzyme substrate test (Colilert®) described in the Standard Methods for Water and Wastewater analysis (American Water Works Association 1995). The Colilert® methodology was adapted for the detection and

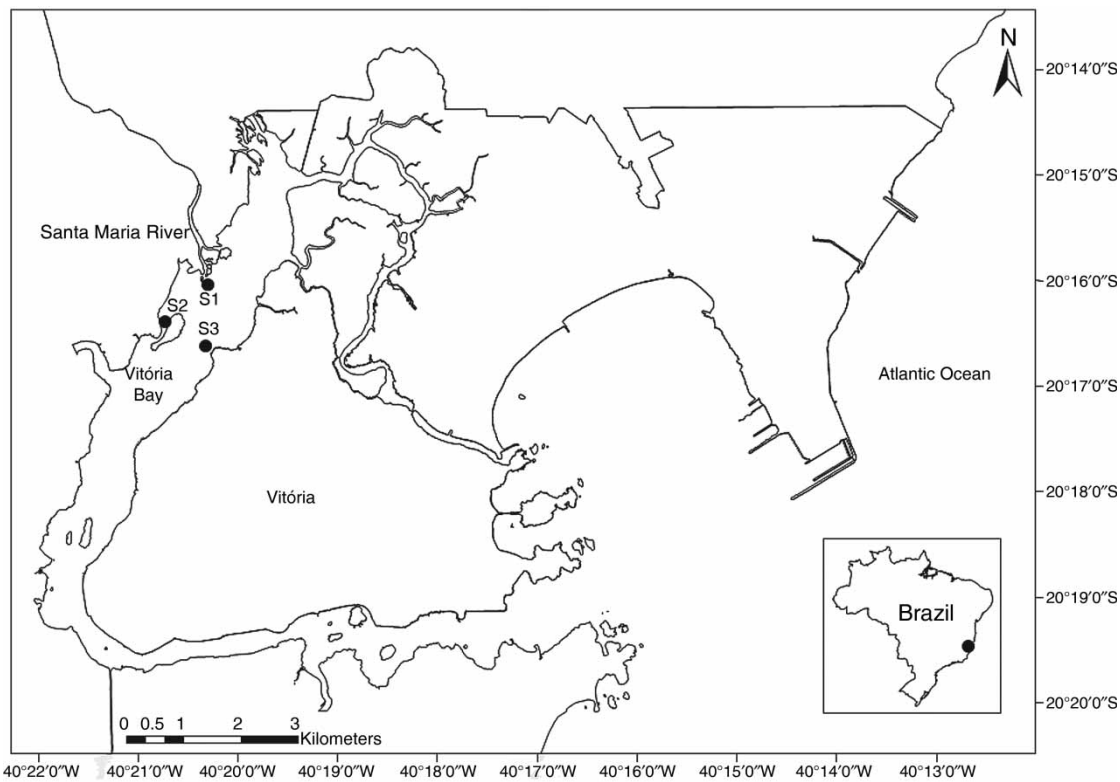

Figure 1 | Sampling locations S1, S2, and S3, in a mangrove region of Vitória Bay, Espirito Santo, Brazil.

quantification of *E. coli* in mussels. Mussels were washed in running water, the gastrointestinal tissue was extracted, and 25 g was weighed and fully homogenized in 225 mL of buffer (KH_2PO_4 0.3 mM, $MgCl_26H_20$ 2.0 mM) using a blender (Walita) in accordance with the methodology recommended by the *Compendium of Methods for the Microbiological Examination of Foods* (American Public Health Association 1992). Ten milliliters of the homogenate was diluted in 90 mL of the same buffer and the following dilutions and steps were done as for water samples.

Viral analysis

The viral analysis of the water samples was evaluated according to the methodology proposed by Katayama *et al.* (2002), with a few changes. One liter of water was mixed with 1 L of Tris/HCl/Ca^{+2} (0.01 M, pH 7.2). After filtration through cellulose ester membrane with a pore size of 1.2 μm (Millipore) to remove larger particles, the filtrate was adjusted to pH 3.5 and filtered again in a cellulose ester membrane with a pore size of 0.45 μm (Millipore). The membrane was rinsed with 200 mL of 0.5 mM H_2SO_4 (pH 3.0) followed by elution with 10 mL of 1 mM NaOH and the eluate was neutralized with 50 mM H_2SO_4 and 100× TE buffer (pH 8.0). The sample was centrifuged in Amicon Ultra-15 tubes (Millipore) at 6,700 × *g* at 4 °C until the sample was concentrated to about 400 μL volume. The sample remained at −80 °C prior to nucleic acid extraction.

Mussels were washed and opened with a sterile knife. Ten grams of digestive tissue were used for viral particle elution following the methods described by Lewis & Metcalf (1988) and Traore *et al.* (1998) with the following modifications: mussel tissue was homogenized in a blender (Walita) with 1:7 (w/v) glycine buffer, pH 9.5 (0.1 M glycine/0.3 M NaCl). After centrifugation at 6,700 × *g* for 30 min at 4 °C the pH of the supernatant was adjusted to 7.5 and the same volume of PEG-NaCl (16%, 0.6 M) was added and incubated overnight at 4 °C. Viruses were recovered by centrifugation at 6,700 × *g* for 30 min at 4 °C and the pellet suspended in Na_2HPO_4 buffer (0.15 M, pH 9.0). The suspension was again clarified by centrifugation at 6,700 × *g* for 30 min at 4 °C and an aliquot of 400 μL of the supernatant was stored at −80 °C prior to nucleic acid extraction.

DNA/RNA extraction and reverse transcription

Two hundred microliters of concentrated sample were used for nucleic acid extraction following the silica method described by Boom *et al.* (1990). Positive controls were used in all extraction rounds. The cDNA synthesis from all samples (water and mussels) were realized according to the protocol proposed by Iturriza-Gomara *et al.* (1999) with modifications as described below. A mixture containing approximately 5 μL of nucleic acid extract and 1 μL of DMSO was denatured at 97 °C for 7 min and kept on ice for 2 min. Then, it was added to a mix containing 1× polymerase chain reaction (PCR) buffer, 200 μM of dNTPs, 2.5 mM $MgCl_2$, 1 μL random primer (pdN6, Amersham, Biosciences) and 60 U of reverse transcriptase superscript II (Invitrogen). The mixture was incubated at 42 °C for 1 hour followed by 95 °C for 10 min. The cDNA was stored at −20 °C for subsequent amplification of specific viruses studied.

PCR amplification, nested and semi-nested PCR

Adenovirus

The amplification of the conserved region of the hexon gene of adenovirus by PCR and nested PCR followed the methodology previously described by Allard *et al.* (2001). The primers Hex1/Hex2 and NeHex3/NeHex4 were used for the first PCR and nested-PCR, respectively. The final mixture consisted of 1× buffer, 200 μM dNTPs, 1.5 mM $MgCl_2$, 0.4 μM of each primer, and 1 U of *Taq* polymerase. The cycle conditions were as follows: 94 °C for 5 min followed by 35 cycles at 94 °C for 20 s, 60 °C for 20 s, and 72 °C for 30 s, and a final extension at 72 °C for 7 min. One microliter of the PCR product was used in a nested-PCR reaction. The same cycling conditions were used for the nested PCR.

Rotavirus

The primers used in the PCR reactions (RV1 and RV2) and nested-PCR (RV3 and RV4), are located in the conserved region of gene VP7 for rotavirus group A, as previously described by Hafliger *et al.* (1997). The PCR final mixture consisted of 1× buffer, 200 μM dNTPs, 1.5 mM $MgCl_2$ (first amplification) or 3.5 mM (nested PCR), 0.4 μM of each

primer, and 1 U of *Taq* polymerase. The cycle conditions were as follows: 1 min at 94 °C followed by 35 cycles at 94 °C for 30 s, 55 °C for 60 s, 72 °C for 60 s, and a final extension at 72 °C for 7 min. One microliter from the first PCR was used in the nested PCR. Forty cycles with the same cycling profile were used for the nested PCR.

Norovirus

The primers used in the PCR reactions (SRII-1 and SRII-2) and semi-nested PCR (SRII-2 and SRII-3), found in the conserved region of the gene for RNA polymerase to genogroup II (GII), were previously described by Hafliger *et al.* (1997). Due to the worldwide prevalence of GII compared with GI, only GII was searched (Lees 2000). The PCR final mixture consisted of 1× buffer, 200 μM dNTPs, 2.5 mM $MgCl_2$ (first amplification) or 2.0 mM (nested PCR), 0.4 μM of each primer, and 1 U of *Taq* polymerase. The cycle conditions were as follows: 5 min at 94 °C followed by 40 cycles at 94 °C for 30 s, 50 °C for 1 min, 72 °C for 30 s, and final extension at 72 °C for 7 min.

Analysis of PCR products

Nested and semi-nested PCR fragments were analyzed by gel electrophoresis. Ten microliters of the amplification product were electrophoresed on 1.8% agarose gels in Tris–borate–EDTA buffer along with a 100 bp DNA ladder (Invitrogen). Fragments were visualized by ethidium bromide staining and UV light.

RESULTS

Water samples ($n = 42$) collected from three sites (S1, S2, and S3) were characterized by their physicochemical parameters: pH, turbidity, electrical conductivity, and total dissolved solids (TDS). Rainfall amount was also recorded during the study. Figure 2 shows the results of turbidity variation and rainfall amount for the 14-month monitoring period. During the summer months (November to February), also known as the rainy season in the southeast of Brazil, there was an increase in the turbidity, mostly evident at S1, which is the site closest to the river. A decrease in electrical conductivity and concentration of TDS were also observed during this period (not shown).

Monitoring of fecal indicators in water and mussels

Figure 3 shows the results of *E. coli* detection during the period of study for the water and mussels collected near

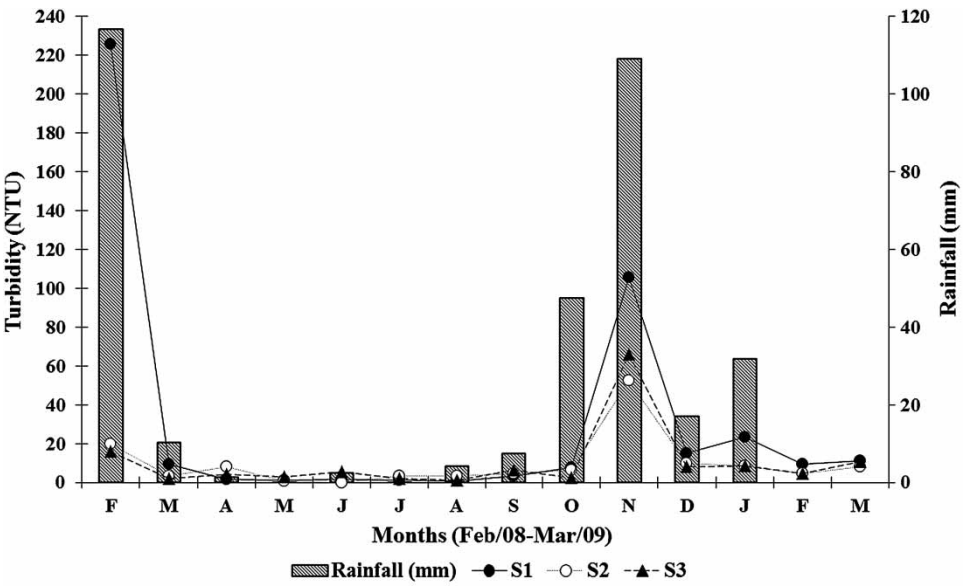

Figure 2 | Monitoring of water turbidity (NTU) and rainfall amount (mm) in S1, S2, and S3 between February 2008 and March 2009.

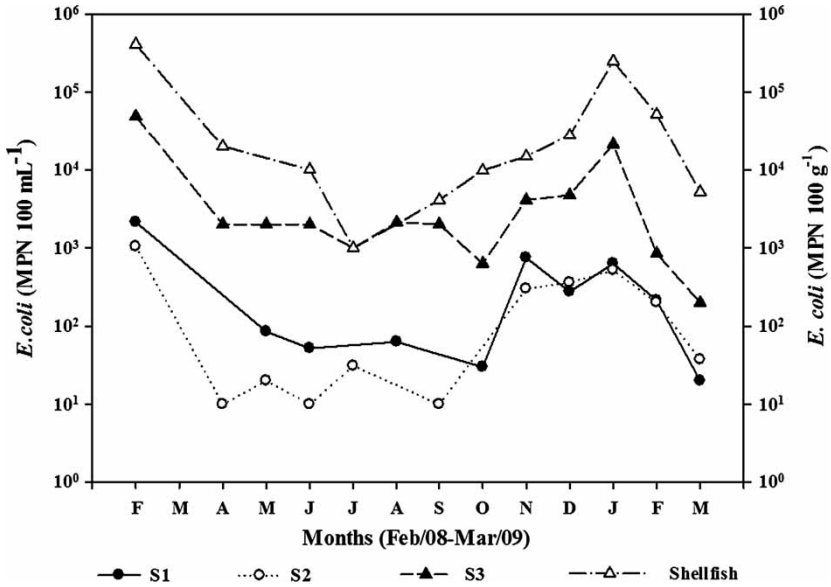

Figure 3 | Monitoring of *E. coli* (MPN 100 mL^{-1}) in water collected at S1, S2, and S3 and mussels (MPN 100 g^{-1}) collected near S2 in the mangrove area between February 2008 and March 2009.

S2. *E. coli* was detected in the water and mussel samples throughout the entire monitoring period. In the summer, there was an increase in bacterial counts at all sites evaluated following the rainfall and turbidity variation. The highest density of *E. coli*, found in February and November of 2008, correlates with the highest amount of accumulated precipitation during the monitoring.

S3, located near the pier and a residential area, was the most contaminated site followed by S1 and S2, respectively. Despite the relatively low concentrations of bacteria recorded in the water samples at S2 during the dry season, mussels collected at this area showed high *E. coli* contamination throughout the period of study.

In the summer, we found that the mussels collected in S2 had about 100 to 1,000 times the average quantity of bacteria detected in 100 mL samples of surrounding waters.

The results of bacterial concentration in the water and mussels collected at all sites can be seen in Table 1.

Detection of viruses in water and mussels

Tables 2 and 3 show the results of PCR detection of viruses in water during the period of study. Norovirus was detected in only one sample from S2 and S3 and was negative in all mussel samples. On the other hand, the adenovirus and rotavirus genomes were detected in 100% of mussel samples.

DISCUSSION

Assessment of the microbiological quality of aquatic ecosystems helps in understanding the impact and consequences

Table 1 | Descriptive statistics of *E. coli* concentrations in water samples collected in S1, S2, and S3, and mussels collected in S2

		Water (MPN 100 mL^{-1})			
Bioindicator		**S1**	**S2**	**S3**	**Mussel (MPN 100 g^{-1})**
E. coli	N	10	11	13	11
	Geometric mean	1.58×10^2	7.20×10^1	2.37×10^3	1.86×10^4
	Median	1.49×10^2	3.74×10^1	2.00×10^3	1.48×10^4
	Minimum	<10	<10	2.00×10^2	1.00×10^3
	Maximum	2.14×10^3	1.05×10^3	4.94×10^4	4.10×10^5

Table 2 | Results of PCR detection of adenovirus (AdV) and rotavirus (RV) in S1, S2, and S3 during 14 months in the mangrove area

	S1		S2		S3	
Month	**AdV**	**RV**	**AdV**	**RV**	**AdV**	**RV**
2008						
Feb	ND	+	+	+	+	+
Mar	+	+	ND	ND	ND	+
Apr	ND	+	+	+	ND	ND
May	ND	+	+	ND	ND	+
Jun	+	+	+	+	+	+
Jul	+	+	+	+	+	+
Aug	+	+	+	+	+	+
Sep	+	ND	+	+	+	+
Oct	+	+	+	+	+	+
Nov	+	+	+	+	+	+
Dec	+	+	+	+	+	+
2009						
Jan	+	ND	+	+	+	+
Feb	+	+	ND	ND	+	+
Mar	+	+	ND	+	ND	+

ND, not detected; +, positive detection.

Table 3 | Adenovirus (AdV), rotavirus (RV), and norovirus (NV) detection frequency in water samples at S1, S2, and S3

Site	Viruses	Number of samples	Number of positive samples	Positive samples (%)
S1	AdV	14	11	78.6%
	RV		13	92.9%
	NoV		0	0%
S2	AdV	14	11	78.6%
	RV		11	78.6%
	NoV		1	7.1%
S3	AdV	14	10	71.4%
	RV		13	92.9%
	NoV		1	7.1%
S1/S2/S3	AdV	42	32	76.2%
	RV		37	88.1%
	NoV		2	4.8%

of inadequate sewage disposal on these ecosystems and on the health of local populations. In this study, the detection of *E. coli* and viral genomes in water samples and mussels collected over a period of 14 months indicated a chronic contamination of the estuary.

According to the standards set by Brazilian legislation relating to quality of water for cultivation of bivalves (Brazil 2005), the study area would be considered inappropriate for harvesting because the recommended limit of 43 MPN $100\,mL^{-1}$ of fecal coliform (or *E. coli*) was exceeded during most of the monitoring period. The adoption of this parameter alone would be enough to indicate that the area is not suitable for cultivation of shellfish. In addition, mussels were approximately 400× more contaminated with *E. coli* than water samples, showing that filter-feeding organisms are more realistic indicators of environmental contamination than circulating water. The presence of the high bacterial densities observed in all mussel samples even when the surrounding waters (S2) presented no restrictions, concurs with other studies that this criterion is not appropriate for shellfish culture and is not enough to guarantee safety regarding pathogenic bacteria and viruses.

One contribution to the microbiological contamination of the estuary comes from the discharge of sewage *in natura* from nearby houses. Additionally, during storm events, runoff from two rivers that flow into the estuary carry many contaminants. The microorganisms can be adsorbed to organic matter, to suspended particles, or in the sediment, contributing to its persistence in the environment (Phanuwan *et al.* 2006; Oliveira *et al.* 2011; Moresco *et al.* 2012). It has been shown that the physicochemical conditions of the environment may favor the persistence of bacteria and viruses in water and that the increases in turbidity may indicate their presence. In this study, we observed that in 2 months of heavy rains (February and November of 2008) the increase of water turbidity was accompanied by an increase in the amount of *E. coli* and in the frequency of positives samples for virus genomes at all water collection sites.

Enteric virus genomes were detected throughout the monitoring period, regardless of the season. The frequency of positive samples for adenovirus and rotavirus was higher during the June to December period when water samples from all three sites tested positive for these viruses. Our results are similar to those described by Le Guyader *et al.* (2000), Miagostovich *et al.* (2008), and Hamza *et al.* (2009), but higher when compared with other studies performed in surface and marine waters (Pina *et al.* 1998; Katayama *et al.* 2002; Hot *et al.* 2003; Choi & Jiang 2005;

Kittigul *et al*. 2005). Norovirus GII was detected only in two water samples using the RT-PCR. Some studies have shown that there may be differences in environmental persistence between viruses belonging to genogroups I and II. Gentry *et al*. (2009) investigated the distribution of norovirus in an estuarine environment and showed that GI and GII represented respectively 90.5 and 9.5% of the positive samples identified. Further studies will be needed to evaluate the prevalence of norovirus genogroup I in our estuarine water samples and mussels. Although mussels are normally cooked before consumption, other seafood collected in the region of study, such as oysters, are consumed raw and may pose a high public health risk.

CONCLUSION

The presence of *E. coli* and enteric virus genomes in water samples and mussels collected in the mangrove area throughout the study indicate that this environment is seriously impacted by continuous discharge of domestic sewage and all shellfish collected in the region should be cooked thoroughly to eliminate the risk of viral illness. Considering that seafood harvesting is one of the principal activities in the region, these results could have social and economic implications for the local population and can be a tool for decision measures of protection and improvement of the environment.

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