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Sanitary quality of edible bivalve mollusks in Southeastern Brazil using an UV based depuration system

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ABSTRACT

The increase in seafood production, especially in mariculture worldwide, has brought out the need of continued monitoring of shellfish production areas in order to ensure safety to human consumption. The purpose of this research was to evaluate pathogenic protozoa, viruses and bacteria contamination in oysters before and after UV depuration procedure, in brackish waters at all stages of cultivation and treatment steps and to enumerate microbiological indicators of fecal contamination from production site up to depuration site in an oyster cooperative located at the Southeastern estuarine area of Brazil. Oysters and brackish water were collected monthly from September 2009 to November 2010. Four sampling sites were selected for enteropathogens analysis: site 1- oyster growth, site 2- catchment water (before UV depuration procedure), site 3 - filtration stage of water treatment (only for protozoa analysis) and site 4- oyster's depuration tank. Three microbiological indicators were examined at sites 1, 2 and 4. The following pathogenic microorganisms were searched: *Giardia* cysts, *Cryptosporidium* oocysts, Human Adenovirus (HAdV), Hepatitis A virus (HAV), Human Norovirus (HnoV) (genogroups I and II), JC strain Polyomavirus (JCPyV) and *Salmonella* sp. Analysis consisted of molecular detection (qPCR) for viruses (oysters and water samples); immunomagnetic separation followed by direct immunofluorescence assay for *Cryptosporidium* oocysts and *Giardia* cysts and also molecular detection (PCR) for the latter (oysters and water samples); commercial kit (Reveal-Neogen®) for *Salmonella* analysis (oysters). *Giardia* was the most prevalent pathogen in all sites where it was detected: 36.3%, 18.1%, 36.3% and 27.2% of water from sites 1, 2, 3 and 4 respectively; 36.3% of oysters from site 1 and 54.5% of depurated oysters were harboring *Giardia* cysts. The huge majority of contaminated samples were classified as *Giardia duodenalis*. HAdv was detected in water and oysters from growth site and HnoV GI in two batches of oysters (site 1) in huge concentrations (2.11×10^{13}, 3.10×10^{12} gc/g). In depuration tank site, *Salmonella* sp., HAV (4.84×10^{3}) and HnoV GII (7.97×10^{14}) were detected once in different batches of oysters. *Cryptosporidium* spp. oocysts were present in 9.0% of water samples from site four. These results reflect the contamination of oysters even when UV depuration procedures are employed in this shellfish treatment plant. Moreover, the molecular comprehension of the sources of contamination is necessary to develop an efficient management strategy allied to shellfish treatment improvement to prevent foodborne illnesses.

1. Introduction

Quality assurance of any kind of food is a critical point for food industries and other sectors linked with food safety. Currently, mariculture is emerging as one of the most profitable cultures in

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terms of both nutritional and economic importance worldwide. The rapid acceleration of this activity in Brazil allied to a lack of environmental sanitation, especially in places where bivalves are harvested, without any standard cleaning procedures, emerges as an important concern related to its cultivation and production (Guiguet Leal and Franco, 2008).

The worldwide distribution of different species of bivalves, their sedentary lifestyle and the efficiency of its filter feeding behavior make these species susceptible to bioaccumulation of pollutants and pathogens, and useful in monitoring aquatic environments (Rippey, 1994; Sauvé et al., 2002).

The risk of occurrence of infectious diseases originated from the consumption of bivalve mollusks has been recognized for several years both by the food industry and health agencies. There is a consensus in the scientific community that if these animals are extracted and consumed from polluted sites, which receive loads of contaminated effluents, there is a maximization of contamination (Rippey, 1994; Potasman et al., 2002).

In the United States, foodborne diseases are responsible for an estimated 76 million illnesses each year and the consumption of bivalve mollusks and other seafood products are implicated in 10–19.0% of these illnesses (Butt et al., 2004).

A wide range of pathogenic microorganisms, which may be present in marine or brackish waters, might be removed and contaminate the flesh of mollusks, representing a threat if ingested raw or undercooked, especially by immunocompromised populations (Fayer et al., 2004; Sincero et al., 2006).

Microorganisms that can be considered a human health risk associated with the ingestion of bivalve mollusks are pathogenic bacteria such as *Salmonella* spp., *Shigella* spp., *Enterobacter* spp. enteropathogenic *Escherichia coli* (O157 strain), *Staphylococcus aureus*, *Yersinia* spp., *Vibrio* spp., *Bacillus cereus* and *Clostridium perfringens*; viruses such as norovirus, rotavirus and hepatitis A virus, and finally parasitic protozoa like *Cryptosporidium* and *Giardia* species and *Entamoeba histolytica* (Lee and Younger, 2002).

In mariculture industry, some measures are adopted as an attempt for purification and elimination of pathogens from mollusks flesh before they are placed onto the market. Some techniques like depuration or transposition are widely used in many countries, including the United States, where mollusks may be kept in tanks or in natural seawater in order to be purified and suitable for human consumption (FDA, 2007).

However, the ideal scenario to minimize health risks associated with shellfish should be the utilization of depuration systems combined with an available disinfection technology. Disinfection with chlorine is not effective against *Giardia* cysts or *Cryptosporidium* oocysts and may be harmful to bivalves; the utilization of ozone has little effect in oocysts and cysts infectivity (Bukhari et al., 2000; Hijnen et al., 2006).

In the shellfish industry, the most commonly used procedure is UV depuration, which has the advantages of being low maintenance, of producing no hazardous by-products to health and of being effective in reducing the pathogens viability. Thus, it is mandatory to reduce contamination prior to human consumption (Hijnen et al., 2006; Eischeid et al., 2009; NSW Food Authority, 2010).

The recovery and isolation of specific pathogens from an environmental sample might pose a challenge because among other factors, they might be present in low concentrations or below the minimum detection limit (Smith and Nichols, 2010). In recent times reliable techniques like immunological methods and molecular biology improved the detection of enteropathogens from shellfish and from environmental samples, which ensured a more accurate diagnosis (Jex et al., 2008; Girones et al., 2010).

In Brazil, public awareness of the risks concerning the ingestion of raw or poorly cooked shellfish must be encouraged and directed to the whole population and, particularly to population categories such as immunocompromised and immunosuppressed individuals.

Likewise, it is necessary to search for human enteropathogens in shellfish commonly eaten raw in various locations around the country and also to verify the efficacy of methods of purification and disinfection used by aquaculture industries in order to ensure safety and absence of microorganisms in edible mollusks destined for human consumption.

The purpose of this research was to evaluate pathogenic protozoa, viruses and bacteria contamination in oysters (*Crassostrea brasiliana*) before and after UV depuration procedure and in brackish waters at all stages of cultivation and treatment steps (UV depuration) in an oyster cooperative located at the Southeastern estuarine area of Brazil. In addition, it was aimed to enumerate microbiological indicators of fecal contamination from production site up to depuration site.

2. Material and methods

2.1. Study site

The oyster cooperative analyzed in this study is located in a huge estuarine complex in “Vale do Ribeira” formed by the confluence of freshwater, waterfalls and seawater in Cananéia city, south coast of São Paulo state, Brazil. Cananéia is one of the most important oyster producing areas in São Paulo and the cooperative is formed by several family members that account for the production of 30–32 thousand dozen oysters per year. About 90.0% of the production is sold by the cooperative all along the north and south coast of São Paulo and in the state capital.

The main product, the native oysters *C. brasiliana*, is produced in a natural and sustainable way in the region. It is destined for the market and represents the main source of income for autochthonous population.

The oysters are primarily grown in a cultivation region known as Mandira (located approximately 25 km away from the cooperative) (25° 00′ 30′′ S and 48° 01′ 29′′ W) in an extractive reserve controlled by the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA) and submitted to a purification and cleansing procedure before they are placed on the market.

In the cooperative (24° 57′ 27′′ S and 47° 54′ 26′′ W) the oysters are kept in plastic trays in a depuration tank, where they will be submitted to UV depuration procedure. The estuarine water is used to fill the depuration tank. This water is sucked by a vacuum pump and filtered through a large sand filter and then passes through cylinders containing UV lamps. Afterwards, the UV treated water is poured into the depuration tank until the animals become submerged. After this step, the oysters are kept in the depuration tank between 3 to maximum 24 h, then removed from the tank and placed on the market.

2.2. Sampling sites

Four sampling sites were elected to search for enteropathogens and microbiological indicators in order to evaluate sanitary quality in all stages of the production of bivalve mollusks. Oysters (*C. brasiliana*) and brackish waters (n = 11samples) were harvested monthly from September 2009 to November 2010.

The following pathogenic microorganisms were searched at specific sites described below: protozoa - *Cryptosporidium* oocysts and *Giardia* cysts (oysters and waters); human viruses - Human Adenovirus (HAdV), Hepatitis A virus (HAV), Human Norovirus (HNoV- Genogroups I and II), JC strain Polyomavirus (JCPyV)

(oysters and waters) and bacteria - *Salmonella* spp. (oyster's samples). Three microbiological indicators of fecal contamination were analyzed in different phases of the production: Thermotolerant coliforms, *Escherichia coli and* Enterococci. Bivalve mollusks and water samples were taken from the following sites:

Site one: oyster growth. Three dozen oysters without treatment, 20 l of water and 100 ml of water.

Site two: Catchment water site (before UV depuration). 20 l of water and 100 ml of water.

Site three: Filter backwash. 10 l of water for protozoan search. Analysis of the sand filter was accomplished by reversing the flow of the pump in order to backwash.

Site four: Depuration tank. Three dozen oysters, 20 l of water and 100 ml of water. Water and oysters samples were collected only after the UV depuration procedure.

2.3. Parasitological examination

2.3.1. Analysis of bivalve mollusks

Oysters were shucked with a sterilized knife. For each sample, oysters were individually dissected to obtain innerwater and gills from 12 animals for *Cryptosporidium* and *Giardia* analysis. The innerwater was aspirated from the shell and animals, sieved and put in 15 ml centrifuge tubes. The contents of the gills were excised and transferred to glass tubes and filled with 15 ml of Tween 80 solution (0.1%). The gill pools were put in a sample mixer (RK Dynal®) and washed for 1 h at 20 RPM. Then, the tubes were vortexed for 15 s, gill tissue was removed and gill wash was sieved and put in 15 ml centrifuge tubes. All tubes containing gill wash pools and innerwater pools were centrifuged twice for 10 min at 1050× *g* to obtain a pellet. The supernatant was discarded and 2 ml of each aliquot of the pellets were stored at 4 °C until purification with immunomagnetic separation (IMS) and direct immunofluorescence assay (IFA).

2.3.2. Analysis of water samples

Water samples derived from four different sites were analyzed for protozoa using the membrane filtration technique, filtered through a 3.0 μm pore size, 47-mm diameter sterile cellulose esters membranes (Millipore®). The membranes were eluted by alternately scraping it with a smooth-edged plastic loop and rinsing it with Tween 80 elution solution (0.1%). The tubes with eluants were centrifuged twice (1050× *g*; 10 min), the pellet was transferred to 2.0 ml plastic eppendorfs and stored until purification with IMS and IFA techniques.

2.3.3. Immunomagnetic separation

All pellet samples comprising innerwater pools, gill wash pools and water samples (pellets ≤ 0.5 ml) were processed by immunomagnetic separation (IMS) using Dynabeads anti-*Cryptosporidium* and anti-*Giardia* kit according to the manufacturer's instructions (Dynal Biotech, Oslo, Norway). During the process of concentration, the IMS bead–parasite complexes were bound to two magnetic holders while the supernatant and debris were discarded during a series of wash steps.

The final step, parasite dissociation from the beads, was performed using heat dissociation. Each sample was submitted to 2 washes of 50 μl of Milli Q water vortexed at the beginning and end of two 10 min incubations in water (80 °C) with the IMS product. The resulting pellet (100 μl) was used to protozoa search where 50 μl was then transferred to a slide well for parasite visualization by direct immunofluorescence assay and 50 μl was used to DNA extraction and molecular analysis of *Giardia*.

2.3.4. Direct immunofluorescence assay

To perform immunofluorescence assay analysis, 50 μl of each IMS product was air dried and then fixed with methanol onto a well slide. A fluorescein isothiocyanate-conjugated with a specific monoclonal antibodies against the cell wall antigens of *Cryptosporidium* and *Giardia* from Merifluor (*Cryptosporidium*/*Giardia* test Kit – Meridian Bioscience Inc., Cincinnati, OH, USA) was used. The oocysts and cysts were identified according to their correct size and shape, pattern and intensity of immunofluorescence staining (bright apple green fluorescence) and a clearly visible (oo) cyst wall. The protozoa were simultaneously confirmed using the fluorogenic vital dye DAPI and phase contrast microscopy to identify internal structures if possible using the microscope Nikon® (50i).

2.3.5. Molecular analysis

Total DNA was isolated by ZR Fungal/Bacterial DNA Kit (Zymo Research). Two fragments of *Giardia* β giardin gene were amplified by PCR. Initially the primers GGL639-658 (5′-AAGTGCGTCAACGAGCAGCT–3′), and GGR789-809 (5′–TTAGTGCTTTGTGACCATCG A-3′) were used to amplify a fragment with 171bp of this gene. The primers GGL405-433 (5′- CATAACGACGCCATCGCGGCTCTCAGGAA-3′) and GGR592-622 (5′-TTTGTGAGCGCTTCTGTCGTGGCAGCGCTAA-3′) generated a 218bp product size of other region from the same gene (Mahbubani et al., 1992).

The two reactions were performed in a volume of 25 μl containing 1× PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 25pmol of each primer, 1.25U Taq Polymerase Platinum Invitrogen and 3 μl of DNA. The negative control was performed with the PCR mix without DNA and the positive control was performed with *Giardia duodenalis* DNA, extracted previously from a positive clinical sample. The PCR was conducted in MJ PTC 100 – MJ Research INC thermocycler. Samples were denatured at 94 °C for 3 min followed by 40 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min. The final elongation was performed at 72 °C for 5 min. The product was confirmed by 3% agarose gel electrophoresis stained with ethidium bromide and visualized under U.V. light on Gene Genius Bio Imaging System Syngene.

2.4. Virological analysis

2.4.1. Analysis of oyster samples

After sampling, oyster shells were opened at the hinge with a sterile oyster knife for the dissection of digestive tissue that was homogenized in a tissue homogenizer Ultra-Turrax (Model T25 basic, IKA Labortechinik). For the recovery of viral particles, the method described by Sincero et al. (2006) was applied. Briefly, the solid-adsorbed viruses were eluted from 2 g of each tissue homogenate (corresponding to tissues derived from 12 dissected oysters) by resuspension in 10 ml of tryptose phosphate broth (TPB) dissolved in 0.05 mol L-1 glycine pH 9.0. The homogenate was purified with an equal volume of chloroform-butanol (1:1) and centrifuged at 13,500× *g* and 4 °C for 15 min. Viruses in the supernatant were precipitated with 12.0% polyethylene glycol (PEG), dissolved in 0.75 mol L-1 NaCl for 2h at 4 °C and centrifuged at 13,500× *g* and 4 °C for 20 min. Each pellet was resuspended in 3 ml of ultrapure water and clarified twice with chloroform (30.0%).

2.4.2. Evaluation of water samples

The method of skimmed milk flocculation procedure was used to concentrate water samples (Calgua et al., 2008). Pre-flocculated skimmed milk solution 1.0% (w/v) was prepared by dissolving 10 g of skimmed milk powder (Difco) in 1 l of artificial seawater. The pH was carefully adjusted to 3.5 using 1N HCl. One hundred ml of this solution were added to each of the previously acidified (pH 3.5) 10 l seawater samples (final concentration of skimmed milk 0.01% (w/v). Samples were stirred for 8 h at room temperature and flocks were allowed to sediment by gravity for another 8 h. Supernatants were carefully removed using a vacuum pump without disturbing

the sediment. The final volume, approximately 500 ml containing the sediment, was transferred to a centrifuge tube and centrifuged (7000× *g*; 30 min) at 12 °C. The supernatant was carefully removed and the pellet resuspended in 8 ml of 0.2M phosphate buffer at pH 7.5 (1:2, v/v of Na_2HPO_4 0.2M and NaH_2PO_4 0.2 M). Once the pellet was completely dissolved, phosphate buffer was added to a final volume of 10 ml. The concentrate was stored at −80 °C.

2.4.3. Quantitative PCR (qPCR)

Viral nucleic acids were extracted by QIAamp Minielute Virus kit (Qiagen®), using 200 μl of the viral concentrate from oyster and water samples, and finally eluted in 60 μl of RNAse/DNAse free water. To reduce the presence of inhibitors of the qPCR reactions, 10 and 100-fold dilutions of RNA/DNA of each sample were carried out. All the extracted DNA/RNA samples were analyzed without dilution and 10-fold diluted and were run in duplicate (4 runs/sample) using the ABI StepOne Plus Real-Time PCR System® (Applied Biosystems, CA, USA). In all qPCRs carried out, the amount of DNA/RNA was defined as the average of the data obtained. A non-template control and an amplification control were included in each run.

For qPCR reactions by TaqMan method, the plasmids with cloned sequences of the viruses HAdV2, HAV, JCPyV were donated by Dr. Rosina Girones (Universitat de Barcelona-ES) and HNoV was donated by Dr. Marize P. Miagostovich (Fiocruz, RJ, Brazil). These plasmids were used as standards for genome quantifications.

All the samples were analyzed in duplicate in the StepOne Plus™ Real-Time PCR System-Applied Biosystems™ using the average for genome copies calculation. Non-template controls were included in each run as negative controls.

The HAdV genome quantification by qPCR was carried out using one assay previously described by Hernroth et al (2002), which had been previously applied by other authors for the detection and quantification of adenoviruses in shellfish and other environmental samples (Formiga-Cruz et al., 2002; Albinana-Gimenez et al., 2009). Amplification was performed in a 25 μl reaction mixture containing 5 μl of diluted NA and 12.5 μl of TaqMan® Universal PCR Master Mix, 0.9 μM of each primer (AdF and AdR) and 0.225 μM of fluorogenic probe AdP1. The following steps were used during the amplification procedure: activation of the uracil-*N*-glycosylase (2 min, 50 °C) and activation of the AmpliTaq Gold for 10 min at 95 °C, 40 amplification cycles (15 s at 95 °C and 1 min at 60 °C).

Quantification of JCPyV genome was carried out using primers and probes previously described by Pal et al., (2006). The PCR mix (10 μM primer and 6 μM probe, working stock solutions) was added to the TaqMan Universal PCR Master mix (Applied Biosystems) for a final concentration of 1 μM primer, 0.6 μM probe for a total reaction volume of 25 μl. The amplification procedure started with an incubation of 2 min at 50 °C followed by 10 min at 95 °C, and 60 cycles of denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 1 min.

For Hepatitis A virus a TaqMan probe described by Jothikumar et al. (2005) was used. The reaction mixture (Ultrasense Probe RT-PCR kit™ Invitrogen, Valencia, CA) was added to 10 μl of the diluted NA sample, 0.25 μM primers and 150 nM fluorogenic probe in a 25 μl final volume. The reverse transcription reaction was followed by 15 min of a hot-start denaturation step and 45 cycles of 95 °C for 10 s, 55 °C for 20 s, and 72 °C for 15 s.

Prior to HNoV amplification by qPCRs, a complementary DNA (cDNA) synthesis was performed by Reverse Transcription (Techne PCR System Thermocycler -Flexigene®) using 5.0 μl of total nucleic acid, Random Primers (Promega™, WI, USA) and M-MLV Reverse Transcriptase (Promega™, WI, USA), according to the manufacturer's recommendations. For HNoV GI and GII detection, primers and probes that amplify RLA-1 or RLA-2 regions of HNoV genome were used. Both reactions were performed in separate tubes by adding 12.5 μl of TaqMan Universal reaction mixture (Applied Biosystems), 3.6 μl of DNAse/RNAse free water and 5.0 μl of cDNA in a 25 μl final volume. The amplification profile included 30 min at 50 °C, 10 min at 95 °C and 40 cycles of 15 s at 95 °C and 1 min at 60 °C (Baert et al., 2008; Kageyama et al., 2003).

2.5. Salmonella examination of bivalve mollusks

The examination of *Salmonella* in oyster flesh was performed according to Albarnaz et al. (2007) using the kit Reveal® *Salmonella* Test System (Neogen, Lansing, MI, USA), according to the manufacturer's instructions. Briefly, 25 g of the oyster flesh homogenate were placed in 200 ml of revive pre-enrichment medium (Neogen, Lansing, MI, USA), for 2 h at 37 °C. After that, 200 ml of enrichment medium selenite cystine (Neogen, Lansing, MI, USA) were added and maintained, for 24 h at 42 °C, under shaking. Finally, five drops of each sample were adsorbed in the test device provided by the manufacturer. The presence or absence of *Salmonella* was verified after 15 min of the adsorption at room temperature. The limit of detection of this kit is 5–10 CFU/25g with a false negative rate of <1% and a false positive rate of <5% (Bird et al., 1999).

2.6. Microbiological indicators

Bacterial analysis was accomplished in decontaminated flasks with capacity of 100 ml. The bacteriological examination was performed using the multiple tubes fermentation technique with appropriate medium to each bacterium and its enumeration was estimated as the most probable number (MPN/100 ml) following the recommendations of Standard Methods for the Examination of Water and Wastewater (American Water Works Association, 2005).

3. Results and discussion

One of the most important aspects of an effective program to manage risks associated with shellfish consumption is the classification of harvest and production areas and the identification of the origin of sources of contamination (NSW Food Authority, 2010).

In Brazil, shellfish production areas are controlled by two main regulations, the Federal Legislation resolution CONAMA N° 357 (National Environmental Council, Brasil, 2005), which contemplates the analysis of thermotolerant coliforms (maximum 43/100 ml) or *E. coli* as a surrogate - in the surrounding water of bivalves cultivation and the Resolution of ANVISA (National Agency of Sanitary Surveillance, Brasil, 2001), which determines in fish or shellfish products the levels of staphylococci coagulase positive and absence of *Salmonella* in 25 g.

The present survey for a wide range of bacteriological indicators and human enteropathogens in shellfish and waters covers all stages of production of oysters destined for human consumption in Brazil and is the first to combine the analysis of bacteria, molecular research of viruses, and waterborne protozoa in real scale to date.

Three microbiological indicators of fecal contamination were analyzed in the oyster growth area (site one) and before and after the UV depuration procedure (site two and four respectively). In the growth area, the microbiological indicators attained low to moderate levels of fecal contamination, especially for thermotolerant coliforms. However, in some months they showed that *E. coli* and *Enterococcus* sp. were almost inexistent (<2.0 MPN/100 ml) in 63.6%, and 45.4% of samples analyzed respectively (Table 1).

The analysis of *Enterococcus* sp. is extremely important because it may signal events of recent fecal contamination and help to elucidate the main sources of fecal contamination, since human and warm blooded animal feces present different proportions of these

Table 1
Microbiological indicators of fecal contamination counts in most probable number, in water samples from all stages related to the production of oysters from Cananéia, São Paulo, Brazil during the 11 months of sampling.

Sample Month/year	Thermotolerant coliforms			*Escherichia coli*			*Enterococcus* sp.		
	Site 1	Site 2	Site 4	Site 1	Site 2	Site 4	Site 1	Site 2	Site 4
S1 September/2009	2.0×10^1	4.0×10^2	2.0×10^1	2.0×10^1	4.0×10^2	2.0×10^1	<2.0	<2.0	<2.0
S2 October/2009	2.0×10^1	2.0×10^1	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0
S3 November/2009	2.0×10^1	2.0×10^1	<2.0	<2.0	<2.0	<2.0	<2.0	6.0	4.0
S4 December/2009	8.0×10^1	<2.0	1.2×10^2	4.0×10^1	<2.0	1.2×10^2	2.3×10^1	<2.0	8.0
S5 January/2010	1.7×10^2	4.0×10^1	2.0×10^1	1.3×10^2	4.0×10^1	2.0×10^1	1.7×10^1	8.0	5.0×10^2
S6 February/2010	1.1×10^2	5.0×10^2	7.0×10^1	7.0×10^1	1.3×10^2	2.0×10^1	<2.0	2.7×10^1	1.7×10^1
S7 April/2010	<2.0	<2.0	1.1×10^2	<2.0	<2.0	<2.0	<2.0	<2.0	4.0
S8 May/2010	<2.0	4.0×10^1	5.0×10^2	<2.0	<2.0	3.0×10^2	2.0	4.0	1.4×10^2
S9 July/2010	$4. \times 10^1$	2.0×10^1	<2.0	<2.0	<2.0	<2.0	8.0	<2.0	1.4×10^2
S10 October/2010	2.6×10^2	3.0×10^2	2.0×10^1	<2.0	<2.0	<2.0	1.7×10^1	1.3×10^2	7.0×10^1
S11 November/2010	<2.0	1.7×10^3	2.0×10^1	<2.0	1.3×10^3	2.0×10^1	2.0	8.0×10^1	5.0×10^1

bacteria (Devriese et al., 1993; Hagedorn et al., 1999). In addition, Enterococci analyses were included in this study since this group of bacteria is considered a good indicator of brackish and seawater quality (Pianetti et al., 2004; Oliveira and Watanabe Pinhata, 2008). The presence of fecal coliforms is widely used to indicate contamination in aquatic environments, however, the association between the occurrence of pathogens and coliforms in surface water is still controversial (Hernroth et al., 2002; Rose et al., 2002).

The microbiological indicators data obtained from site two (catchment water) and site four (depuration tank) in several occasions presented higher densities of bacteria when compared to site one (Table 1) and indicated poorer water quality in the final and most important phases of the treatment, signaling that they would be more prone to contamination.

The cooperative of oysters is located 6 km away from Cananéia city. For this reason, water supplied by companies and treatment sewage plants are not available in that region. Additionally, there is a big tourist club hotel located really close to the cooperative and both are within a few meters from the estuary. The water supply is provided by wells and sewage is deposited in septic tanks for treatment.

Even though the water of the depuration tank (site 4) is submitted to filtration and disinfection with UV, it may occasionally attain higher levels of fecal coliforms, in comparison with the original estuarine water (site 2), which was attested in different months of sampling for specific bacteria (Table 1), especially in December (2009) and May (2010) for all the indicators analyzed.

This fact might be justified by the presence of thousands of oysters that are under depuration process and may contribute to contamination of depuration water. Furthermore, this condition denotes fragility in the purification process of oysters since these animals are kept very briefly under depuration, and the water is not changed during the process and especially because these animals can be harboring pathogens which will not be exposed to UV light, and thus may be released in depuration water tank contributing to the spread of contamination.

The overall analysis for pathogenic microorganisms in water and oysters from the growth area (site 1) revealed absence of *Cryptosporidium* spp. *Salmonella* sp. and for the majority of viruses. *G. duodenalis* were detected in 36.3% of water samples (Table 2) and in the same proportion of oysters samples (3 positive samples in gill wash and one in innerwater pool) (Table 3).

Human Adenovirus (HadV) was present in water samples from site 1 in October, 2009, May, 2010 and in July, 2010 (Table 2), where very low levels of thermotolerant coliforms, *E. coli* and *Enterococcus* sp. were detected (Table 1) and it were also detected in 36.3% ($n = 4$ batches of oysters) cultivated at this site (Table 3), attesting the contamination of the cultivation area by human feces.

Among enteric viruses widely distributed in aquatic environments, the HadV presents higher stability under environmental stressors, like UV-C irradiation, temperature and pH oscillation, to physical-chemical disinfection procedures and also to sewage treatment procedures (Calgua et al., 2008).

The qPCR analysis detected a significant large quantity of norovirus GI in oysters from the growth area (2.11×10^{13} and 3.10×10^{12}) in two different occasions (Table 3). The norovirus from genogroup I are primary pathogens associated with shellfish-borne outbreaks and its transmission through shellfish consumption is

Table 2
Results of water samples for protozoa and viruses on different sites related to the production of oysters from Cananéia, São Paulo, Brazil during the11 months of sampling with at least one positive finding of any pathogen on specific sites.

Sample Month/Year	*Giardia duodenalis*				*Cryptosporidium* spp.	Human Adenovirus
	Site 1	Site 2	Site 3	Site 4	Site 4	Site 1
S1 September/2009	A/ND	NS	A/ND	P^+	A^-	N
S2 October/2009	A	P^*	P^*	P^*	A^-	2.82×10^2 gc/l
S3 November/2009	P^*	P^*	P^*	A	A^-	N
S4 December/2009	P^*	A	A	A	P^{**}	N
S5 January/2010	A	A	A	A	A^-	N
S6 February/2010	P^*	A	A	A	A^-	N
S7 April/2010	P^*	A	P^*	P^+	A^-	N
S8 May/2010	A	A	A	A	A^-	7.10×10^7 gc/l
S9 July/2010	A	A	A	A	A^-	3.02×10^7 gc/l
S10 October/2010	A/ND	A/ND	P^{**}	A/ND	A^-	N
S11 November/2010	A/ND	A/ND	A/ND	A/ND	A^-	N

A: Absence on IFA and PCR; A/ND: Absence on IFA and PCR not done; NS: No sample; P^+: Positive on IFA and PCR; P^*: Positive on PCR for *Giardia duodenalis*; P^{**}: positive on IFA for *Giardia* spp. or *Cryptosporidium* spp.; A^- Absence on IFA; N: Negative; gc/l: genome copies/liter.

Table 3
Results of oyster's samples for protozoa, viruses and *Salmonella* sp. on different sites related to the production of oysters from Cananéia, São Paulo, Brazil during the 11 months of sampling with at least one positive finding of any pathogen on specific sites.

Sample Month/Year	Oysters from growing area – Site one				Oysters from depuration tank – Site four				
	Giardia duodenalis		Human Adenovirus	Human Norovirus (GI)	Giardia		Hepatitis A	Human Norovirus (GII)	Salmonella
	Gill wash	Innerwater	Digestive tissue	Digestive tissue	Gill Wash	Innerwater	Digestive tissue	Digestive tissue	25 g of tissue
S1 September/2009	A/ND	A/ND	N	N	A/ND	P**	N	N	N
S2 October/2009	A	P*	N	N	P*	A	N	7.97×10^{14}	N
S3 November/2009	A	A	N	N	P*	A	N	N	N
S4 December/2009	A	A	3.32×10^{3} gc/g	N	A	P*	N	N	N
S5 January/2010	A	A	N	N	P*	A	N	N	P***
S6 February/2010	P*	A	9.55×10^{6}	N	A	A	N	N	N
S7 April/2010	P*	A	2.05×10^{6}	N	A	A	N	N	N
S8 May/2010	A	A	N	N	A	A	4.84×10^{3}	N	N
S9 July/2010	P*	A	N	2.11×10^{13} gc/g	A	P*	N	N	N
S10 October/2010	A/ND	A/ND	N	3.10×10^{12} gc/g	A	A	N	N	N
S11 November/2010	A/ND	A/ND	1.30×10^{8}	N	A	A	N	N	N

A: Absence on IFA and PCR; A/ND: Absence on IFA and PCR not done; P*: Positive on PCR for *Giardia duodenalis*; P**: positive on IFA for *Giardia* spp.; P***: positive on bacterial culture; N: Negative; gc/g: genome copies/gram.

asseverated by its ability to remain in the oyster's digestive tract for various weeks (Lees, 2000; Le Guyander et al., 2006). Several studies showed that GI norovirus is frequently detected in environmental samples; norovirus related disease and most outbreaks occur more frequently during the colder months, which is in accordance with our findings (Katayama et al., 2008; Atmar, 2010; Wyn-Jones et al., 2011).

Cryptosporidium oocysts were not detected in any water samples derived from site two (Catchment water) and site three (Filter backwash). *G. duodenalis*-specific PCR was performed and it confirmed the presence of this pathogen in these samples- (site 2, 18.1% and site 3, 36.3%) (Table 2). On site 3, four samples were positive for this parasite (3 by PCR and one by IFA). The analysis of sand filter is quite important because a huge amount of water is filtered before being poured into the depuration tank and in the present study, it was shown that water from site two is under influence and vulnerable to contamination by human or animal feces. The sum of the findings of sites two and three demonstrated that the contamination by *Giardia* seems to be frequent, reinforcing the importance of continued monitoring of pathogens in all stages of production. Additionally, the evaluation of sources of fecal loading using molecular techniques is extremely necessary to a better understanding of environmental contamination and also as a key to predicting more accurately real risks for human health.

For protozoa parasites, which naturally exhibit bigger sizes than viruses and bacteria, the phase of filtration may function as a barrier; however, *Cryptosporidium* oocysts are considered notoriously robust and have diminutive size and evade filtration by compressibility (Robertson and Gjerde, 2007).

Viruses, bacteria and protozoa may contaminate water and oysters at the depuration tank through evading filtration or being eliminated by oysters during the depuration process, since animals may be previously contaminated in the growth phase of production or might be contaminated when they are allocated in site two, because the oysters that are not sold are put in the estuary to obtain nutrients until they are submitted to a new depuration process before being placed on the market.

If UV disinfection is not able to destroy pathogens infectivity or if no disinfection occurs during depuration, viable pathogenic organisms may be removed by oysters posing a threat to public health whenever they are consumed in a raw or lightly cooked form.

Water used for depuration must be free from any contamination. When ultra-violet disinfection lamps are used to treat the water, it must be disinfected continuously during the depuration process according to Australian Regulation NSW Food Authority, (2010) and shellfish must be depurated for at least 36 h.

While monitoring the sanitary quality of bivalve mollusks, different pathogens were detected exactly in the final stages of the treatment of animals during depuration in water and oysters, which is a worrying sign. From oyster samples, 9.0% was contaminated with *Salmonella* sp., which according to the current Brazilian legislation is not suitable for human consumption.

Among all pathogens found in oysters after the UV depuration treatment, *Giardia* was the most prevalent, since 54.5% ($n = 6$ samples of oysters) were harboring this parasite and 5 samples were confirmed as *Giardia duodenalis* (Table 3).

Virus contamination of oysters was also evidenced in oysters from the depuration tank to hepatitis A virus (HAV) and human norovirus GII (Table 3). Both viruses are often found in fecal contaminated waters and are responsible for many oyster-associated enteric diseases in humans (Lees, 2000; Pintó et al., 2010; Atmar, 2010). Considering that these viruses were found in great numbers, since other genogroup of norovirus were detected in oysters from the growth area, and finally that enteric viruses were not found in any month at sites two and especially site four, it might be possible that this contamination occurred in the growth area and persisted until the oysters were allocated at the UV depuration treatment step.

Protozoa parasites were the only pathogens found in the depuration water tank: 27.2% ($n = 3$ samples) were contaminated with *Giardia duodenalis* cysts and in 9.0% ($n = 1$ sample) it was observed the presence of *Cryptosporidium* oocysts (Table 2). The occurrence of protozoa parasites in water and oysters from the depuration tank site could be interpreted as a risk of acquiring these foodborne diseases if they are found to be species infectious to humans. In addition, this finding represents a serious concern to public health considering that low infective doses of *Cryptosporidium* oocysts and *Giardia* cysts may establish an infection, with the possibility, in the case of cryptosporidiosis, of development of disease by the ingestion of a single oocyst (Rose, 1997).

PCR assay revealed that DNA extracted from *Giardia* cysts, detected in different water sites and oysters have the potential to infect humans since they were classified as *Giardia duodenalis*. However, the elucidation of this hypothesis will be confirmed with sequencing reactions, the next step of this research.

One of the most important findings of this study consisted in the amplification of target DNA sequences of *Giardia duodenalis* in all stages of production of oysters, which occurred in sample 2, where

the protozoa was present in innerwater pool of oysters from site 1, on water samples from site 2, 3 and 4 and in gill wash pool of oysters from site 4 (Tables 2 and 3). This particular result showed that contamination by this pathogen occurs at the same time in different parts of this estuarine environment and even more intriguing is the widespread distribution of this waterborne pathogen through the estuary if considered that growth area (site 1) is located 25 km away from the cooperative.

Giardia, which has a wide distribution in the environment, is an important causative agent of waterborne and foodborne outbreaks and is considered the most common enteric protozoan pathogen of humans, domestic animals and wildlife (Thompson, 2004; Monis et al., 2009).

The overall analysis for different types of virus contamination showed that more positive samples were found in oysters when compared to water samples and this may have been due to the influence of viral particles to dispersion and dilution that naturally occur in lotic aquatic environments or by exposure to environmental stressors (Brookes et al., 2004; Wyn-Jones et al., 2011).

A previous study, conducted in the same estuarine area in Cananéia, demonstrated the presence of pathogenic bacteria in oysters harvested before and after ozone treatment. Even though the treatment of water with ozone was considered important to reduce contamination, the authors pointed out that some batches of treated oysters were not approved for the market according to Brazilian standard legislation (Ristori et al., 2007).

In the present study, the treatment with UV depuration must be considered the most important stage of the production of oysters because it precedes human consumption. However, numerous studies revealed that totally removing or inactivating pathogens from oyster tissue may pose a real challenge.

Sunnotel et al. (2007) evaluated the effectiveness of standard UV depuration at inactivating *Cryptosporidium parvum*, a zoonotic species, recovered from spiked oysters and found out that the treatment with UV at full power (50 W per pass) in water continuously exposed to UV for 48 h resulted in the largest degree of oocyst inactivation ($P < 0.05$; 13-fold), nonetheless, the treatment was not able to inactivate all viable parasites recovered from oyster tissues.

Gómez-Couso et al. (2003) verified the presence of *Cryptosporidium* spp. oocysts in 46.2% of mollusks samples subjected to depuration for 6 and 8–14 days and despite depuration time, 50.0% of the contaminated samples contained viable oocysts. Graczyk et al. (1999) demonstrated that clams (*Corbicula fluminea*) exposed to cysts of *Giardia duodenalis* retained these cysts in their tissue for at least two and three weeks following two artificial experiments and since *G. duodenalis* cysts were not detected in clam feces it was unlikely that the animals were re-exposed to the pathogen.

Even though shellfish could be able to release pathogens in depuration water, they might be taken up by other co-existing oysters (Gómez-Couso et al., 2001). Similarly, this fact must be taken into account in our study since the depuration occurs in a static system and thus contributes to the spread of contamination.

Human norovirus and Hepatitis A virus need a long time of depuration to be completely eliminated; these viruses were retained in *Crassostrea ariakensis* after 29 days of depuration and in *Crassostrea virginica* after 14 and 29 days respectively (Nappier et al., 2009). In the present study, further analyses are now in process in order to check virus infectivity after the depuration treatment step. Factors that could be pointed to the inclusion of monitoring these pathogens by the Brazilian legislation in shellfish cultivation areas would be: the low infective dose of *Cryptosporidium* and *Giardia*; the opportunistic nature of *Cryptosporidium*; the ubiquitous and high endemic prevalence of *Giardia* in Brazil; the long term survival of protozoa in marine or estuarine environment (which ranges from several months to one year), the remarkable resistance of protozoa and Adenovirus to environmental stressors and to several disinfection technologies; the emerging importance of norovirus as a major causative agent of gastroenteritis outbreaks, including shellfish associated outbreaks; the inefficiency of the depuration process in totally eliminating foodborne and waterborne viruses and protozoa (Rose, 1997; Tamburrini and Pozio, 1999; Smith et al., 2007; Eischeid et al., 2009; Atmar, 2010).

Other studies must be conducted as an attempt to improve the dynamics of different pathogens released during depuration and also its inactivation, with measures that involve increasing depuration time, with water circulation and continuously exposure to UV treatment.

In conclusion, these results reflect the contamination of shellfish even when UV depuration procedures are utilized in this shellfish treatment plant and highlight the need of continued monitoring pathogens and suit the legislation of the cultivation of shellfish in Brazil. The evaluation of origin of sources of pollution is quite important because may be applied as a management strategy to prevent or mitigate fecal contamination into the estuarine environment and provide valuable information to the knowledge of risk assessments of many infections caused by different pathogens linked with shellfish consumption.

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