Possible health impacts due to animal and human fecal pollution in water intended for drinking water supply of Rio de Janeiro, Brazil

Kayo Bianco, Rodolpho Mattos Albano, Samara Sant'Anna de Oliveira, Ana Paula Alves Nascimento, Thaís dos Santos and Maysa Mandetta Clementino

ABSTRACT

Fecal matter is considered as one of the worst pollutants in waterbodies due to the potential spread of waterborne diseases. This study aimed to determine the host-specific fecal contamination in two Brazilian watersheds and to predict the possible impacts on human health. Fecal sources were enumerated using host-specific genetic markers to swine (16S rRNA), human and bovine (archaeal *nifH*), and equine (archaeal *mcrA*). A single cycling condition was established for four markers aiming to decrease the analysis time. Fifteen samples from São João watershed (75%) and 25 from Guandu (62.5%) presenting *Escherichia coli* enumeration in compliance with Brazilian guidelines (<1,000 MPN/100 mL) showed the human marker. Furthermore, the bovine, swine, and equine markers were present in 92% (59/64), 89% (57/64), and 81% (52/64) of the water samples, respectively. The molecular markers proposed for qPCR in our study were sensitivity and specific enough to detect host-specific fecal pollution in all samples regardless of *E. coli* levels reaffirming the low correlation among them and supporting their use in water quality monitoring programs. To our knowledge, this is the first study using this approach for quantification of *nifH*, *mcrA*, and *rrs* gene-associated human and animal fecal pollution in waters intended for drinking water supply in Brazil.

Key words | archaeal marker, bacterial indicator, drinking water supply, fecal pollution, microbial source tracking, water quality

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INTRODUCTION

Water pollution through wastewater is an increasing problem that can compromise both aquatic wildlife and human health, due to the high levels of intestinal pathogens from human and animal sources. These pollutants, transported by rain runoff, affect watersheds, causing eutrophication, carrying sediments and introducing pathogenic microorganisms. Rural waste is one of the main sources of fecal contamination residues in aquatic ecosystems due to the high number of commercial farms of cattle, goats, horses, pigs, and poultry (Gómez-Doñate *et al.* 2016).

In addition to biological contamination caused by fecal matter, discharges from mining, as well as industrial and agricultural activities, contribute to increased levels of several pollutants in aquatic ecosystems, which may pose a threat to human health (Adeogun *et al.* 2016; Cao *et al.* 2017). The monitoring of water quality for public supply is important, in particular because some microorganisms can resist standard water treatment procedures (Tessler *et al.* 2017).

Worldwide, the enumeration of fecal indicator bacteria (FIB), such as *Escherichia coli*, in waterbodies is the

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standard methodology adopted for water quality monitoring programs (Conselho Nacional do Meio Ambiente 2005, 2011; USEPA 2012, 2017). Without a doubt, the assessment of water quality using FIB has been a valuable contribution to water quality management since the late 19th century (Tallon *et al.* 2005; Altenburger *et al.* 2019). However, studies have suggested that these indicators may also be present in non-enteric environments such as sediments and soil (Desmarais *et al.* 2002; Whitman *et al.* 2003; Byappanahalli *et al.* 2012; Hassard *et al.* 2016). In addition, this methodology presents another limitation, the absence of discriminatory power, that is, the ability to relate fecal contamination to the source. This shortcoming makes it difficult to associate FIB levels with real population health hazards (Rochelle-Newall *et al.* 2015). Consequently, the determination of fecal pollution sources is fundamental for a more accurate assessment of a possible negative impact on public health (Raith *et al.* 2013; Bianco *et al.* 2015; Henry *et al.* 2016).

Currently, microbial source tracking (MST) has been adopted as an alternative to traditional methods for fecal pollution, based upon cultivation and enumeration of FIB (Unno *et al.* 2018). MST tools are used to associate the presence of specific markers, as microbes or phages, with a particular host responsible for fecal contamination (Ahmed *et al.* 2018). Due to their higher abundance compared with traditional indicators, inability to grow in extra-enteric environments and high specificity, several anaerobic microorganisms such as *Bifidobacterium* spp., *Clostridium perfringens*, members of the order *Bacteroidales* and *Methanobrevibacter* spp. are proposed as specific marker genes of fecal contamination (Ufnar *et al.* 2006; Hughes *et al.* 2017).

Human fecal pollution markers of the *Bacteroidales* 16S rRNA and non-16S rRNA genes are highly host-specific, although they may occasionally be present in non-human fecal specimens, which may lead to detection of false positives (Weidhaas *et al.* 2010; Ahmed *et al.* 2016). In fact, the most commonly used marker, *Bacteroidales* HF183, is not fully specific for human fecal pollution, although it has some advantages such as being widely distributed in the human population and being present at relatively high levels in wastewater (Ahmed *et al.* 2009).

A study investigated the sensitivity and specificity of 16S rRNA genes of host-specific *Bacteroidales* species using the HF183F and CF128F primers in France, Ireland, Portugal, and the UK to evaluate their usefulness in determining the origin of fecal pollution (Gawler *et al.* 2007). It was shown that the HF183F marker displayed high sensitivity (80–100%) and specificity (91–100%). On the other hand, the CF128F marker displayed 100% sensitivity in all four countries. However, strong regional variations in specificity (41–96%) were observed, highlighting the need for local validation before this marker is employed in source tracking of fecal contamination (Gawler *et al.* 2007). In this way, in a previous study, we evaluated fecal markers of the Archaea and Bacteria domains and verified the prevalence of archaeal *nifH* and *mcrA* genes associated with human, bovine, and equine fecal contamination in the watersheds analyzed (Bianco *et al.* 2015).

The members of the genus *Methanobrevibacter* are fastidious archaeal microorganisms and obligate anaerobes that belong to the order *Methanobacteriales*. Fifteen known species are found in animal intestinal tracts, anaerobic sludge, and sewage treatment plants (Lai *et al.* 2004; Horz & Conrads 2011). As only a few species occur in more than one host and are associated with factors like nutritional restrictions and oxygen stress conditions that limit their survival in extra-enteric environments, they could be seen as specific microbial indicators of fecal recent pollution in environmental samples (Ko *et al.* 2018).

Brazil has one of the largest freshwater reserves in the world (12%). Although approximately 85% of the Brazilian population has access to potable water, about 43% of Brazilian cities still do not have a sewage system and, as a whole, only 45% of Rio de Janeiro State sewage is treated (Almeida *et al.* 2018). The Lagos region water supply system, in the north of Rio de Janeiro State, receives water from the Juturnaíba Dam, formed by São João, Bacaxá, and Capivari Rivers (Wasserman *et al.* 2018). Despite the importance of the São João watershed, there are no government agencies responsible for assessing the quality of these water bodies.

The water supply system for the metropolitan region of Rio de Janeiro, the second largest region of the country and the third largest in South America, with a population of about 12.6 million inhabitants, is provided by Guandu watershed. Its main influents are the Macacos, Santana, Piraí, Poços, and Queimados Rivers, which are negatively

impacted by agricultural, domestic, and industrial sewage discharge (Branco & Guarino 2012). The low percentage (45%) of sewage treatment in Rio de Janeiro State and the disordered human occupation near these watersheds have promoted the deterioration of waters, increasing the dissemination of waterborne diseases (Britto *et al.* 2016).

This study aims to monitor fecal pollution in watersheds intended for drinking water supply through bacterial/archaeal domain host-specific marker genes by qPCR. To our knowledge, this is the first study using this approach for quantification of nifH (bovine-associated *Meth. ruminantium* and human-associated *Meth. smithii*), *mcrA* (equine-associated *Meth. gottschalkii*), and *rrs* (swine-associated *Bacteroidales*) genes as human, bovine, equine, and swine fecal markers of pollution in waters intended for drinking water supply in Brazil.

Monitoring based on MST significantly enhances the knowledge of the origin of microbial fecal pollution patterns in aquatic environments. It could be a powerful tool to guide the management of water quality for human consumption, contributing to the implementation of accurate molecular techniques in the Brazilian guidelines.

MATERIAL AND METHODS

Fecal sampling

To evaluate the host specificity and sensitivity of gene markers used for qPCR in this study, 116 fecal samples were utilized from six different hosts. Human feces ($n = 25$) were obtained from volunteers from different locales of Rio de Janeiro. Horse ($n = 20$), swine ($n = 17$), sheep ($n = 14$), chicken ($n = 19$), and bovine ($n = 21$) feces were obtained from several breeding farms from distinct regions of Brazil. Fresh stools of each animal sample (2–20 grams) collected in sterile Falcon tubes were refrigerated until arrival at the laboratory.

Environmental water sampling

São João river watershed

This watershed is composed mainly of Bacaxá, Capivari, and São João Rivers that flow to the Juturnaíba Dam. Two collection points were selected in the São João River, one before (22°35′S/41°59′W) and another after (22°35′/41°59′W) the dam. Two points in the Capivari River, both before the dam, one before (22°38′S/42°24′W) and another after the city (22°38′S/42°22′W), were selected. Finally, one point in the Bacaxá River (22°38′/42°22′W) and one point in the Juturnaíba dam (22°38′S/42°18′W) near the capitation point of the water treatment plant were also selected (Figure 1).

Guandu river watershed

Guandu river watershed is composed of seven influents, two dams (Santa Cecilia and Guandu Dam) and the Guandu Lagoon that flows into the Guandu River. It is affected by domestic and industrial waste and is the major source of drinking water supply to the Rio de Janeiro metropolitan region (21 cities), Brazil. The samples from the Guandu River watershed were collected at ten points before the Guandu Water Treatment Station capitation: Santa Cecilia Dam (22°28′S/43°50′W), Piraí River (22°37′S/43°53′W), Ribeirão das Lajes Dam (22°41′S/43°51′W), Macacos River (22°38′S/43°42′W), Santana River (22°38′S/43°40′W), Guandu River (22°43′S/43°38′W), Poços River (22°45′S/43°36′W), Queimados River (22°45′S/43°36′W), Guandu Lagoon (22°47′S/43°37′W) and one point where the water is collected for treatment named the Guandu Dam (22°48′S/43°37′W) (Figure 1).

Two samples were collected in spring (November 2013 and 2014) and two samples in autumn (May 2014 and 2015) from 16 collection points (11 rivers, 4 dams, 1 lagoon) from the two watersheds studied. Samples (5.0 L) were taken from surface water (15 to 20 cm) in polyethylene bottles. Physical and chemical parameters, temperature, pH, conductivity, dissolved oxygen (DO), turbidity, and salinity of the samples were analyzed using Water Quality Checker U-10 (HORIBA). All samples were kept refrigerated and processed at the laboratory within 4 hours. The variation significance of the physicochemical parameters was determined by Grubbs' test with a significance level of 5% ($\alpha = 0.05$) under the condition of unilaterality. For statistical treatment the outliers package of software R version 3.4.1 for Windows was used.

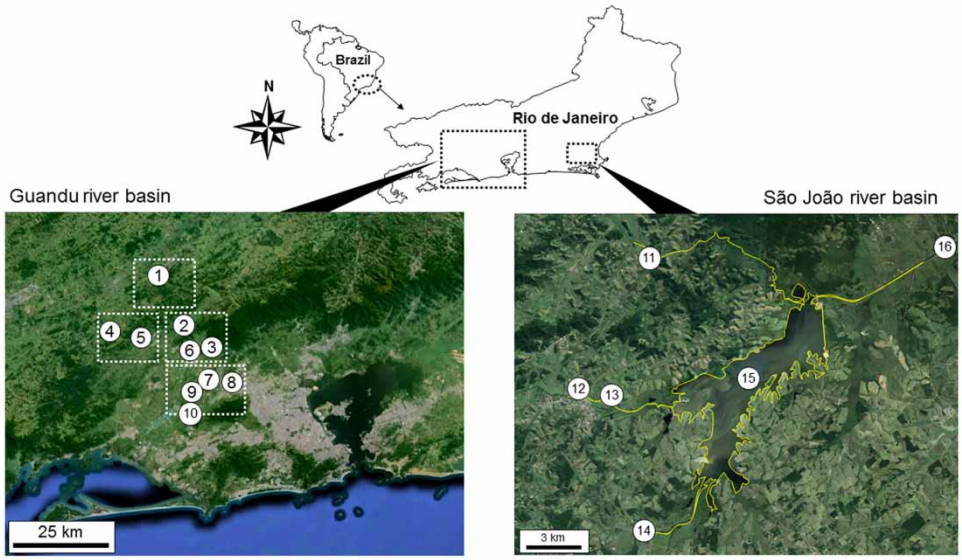

Figure 1 | Geographical location of the São João and Guandu watersheds in the state of Rio de Janeiro, Brazil: (1) Santa Cecilia Dam; (2) Piraí River; (3) Ribeirão das Lajes Dam; (4) Macacos River; (5) Santana River; (6) Guandu River; (7) Poços River; (8) Queimados River; (9) Guandu Lagoon; (10) Guandu Dam; (11) São João River; (12) Capivari River; (13) Capivari Railway; (14) Bacaxá River; (15) Juturnaíba Dam; (16) mouth of the São João River.

Enumeration of FIB

The enumeration of total coliforms and *E. coli*, alternatively thermotolerant coliform, according to Brazilian water quality standards (CONAMA 2005) was evaluated by the defined substrate method (Colilert, IDEXX) (Baird *et al.* 2012). The water samples were diluted (1/10) and retested to determine the bacterial concentrations more accurately.

Water and fecal sample processing and DNA extraction

Approximately 0.5 g of fecal material was suspended in 1 mL of phosphate-buffered saline (PBS) (0.12 M, pH 8.0). The diluted fecal sample was mixed on a rotating platform to produce a homogeneous suspension and stored at −20 °C until use. The water samples (500 mL) were concentrated by filtration through the Stericup® system (Millipore) (0.22 μm) for MST analysis. DNA extraction from both water and homogenous fecal suspension samples was done according to a modified version of previously described protocols (Ogram *et al.* 1987; Bianco *et al.* 2015). Briefly, the water sample filters were submitted to freezing and thawing (−70 °C/2 min, 65 °C/2 min). Then, glass beads (0.1-mm diameter) were added and the suspension was shaken in a Bead-Beater (BioSpec, Bartlesville, USA). The DNA was extracted with phenol–chloroform [1:1 (v/v)] and chloroform–isoamyl alcohol [24:1 (v/v)]. In addition, the DNA was purified using the Dnaeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany) to remove possible PCR inhibitors. The purified DNA was quantified with a Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions and verified on 0.8% agarose gel electrophoresis with GelRED™ (Biotium, USA) staining.

Probe and primer design for qPCR assays

The sets of probes and primers for qPCR assays were developed to quantify 16S rRNA gene copies of swine-associated *Bacteroidales*, *mcrA* gene of *Meth. gottschalkii* equine-associated, and *nifH* gene of *Meth. smithii* and *Meth. ruminantium* (human- and bovine-associated, respectively). Initially, primer and probe designs were based on qPCR conditions according to Johnston *et al.* (2010). Primers were designed with a melting point (Tm) between 57 °C and 60 °C and probes with a Tm 7 °C to 10 °C higher than that of the primers and were purchased from Integrated DNA Technologies (Coralville, IA, USA). Primer and probe sets for qPCR analysis were designed from published *nifH*, *mcrA*, and 16S rRNA gene sequences (CP000678;

Table 1 | Primer and probe sequences designed for qPCR

Primers	Sequence (5′–3′)	Target	Product size (bp)
qMnif12F	CAGTGAAGAGGATATTATTGTA	*nifH*/Human-associated *Methanobrevibacter smithii*	131
qMnif143R	ACACCTAAGTTTTCAAGTC		
qMnifProbe	(FAM)AGCTACTATTACACCACGTCCG(BHQ – 1)		
qMru181-F	ATGTGTTGAAAGCGGAGGTC	*nifH*/Bovine-associated *Methanobrevibacter ruminantium*	145
qMru325-R	GCAGACCACATCCCCTAAAA		
qMru201-P	(Cy5)CTGAACCTGGAGTGGGATGT (BHQ – 2)		
qPF191F	CAGCAGTGAGGAATATTG	*16S rRNA*/Swine-associated *Bacteroidales*	86
qPF276R	GCAGTTTACAACCCATAG		
qPFProbe	(TET)CACGCTACTTGGCTGGTTCA(BHQ – 1)		
qGot23F	GGTACTTATCCATGTACTTAC	*mcrA*/Equine-associated *Methanobrevibacter gottschalkii*	80
qGot103R	CACCACATTGATCTTGTAA		
qGotProbe	(Cy3.5)CGAAACCGTAGAAACCTAATCTGGAAT(BHQ – 1)		

AB019137; EU919431; KM924826) using the Oligo Architect Online™ software (http://www.oligoarchitect.com) (Table 1). Subsequently, the cross-reactivity was evaluated *in silico* using BLASTn searches against GenBank (NCBI).

The qPCR assay was tested first on a panel of closely related methanogen species, to ensure that there was no cross-reactivity between genetically similar organisms (*Meth. smithii* DSM 11975, 2374, 2375, and 861, *Meth. ruminantium* DSM 1093, *Meth. acididurans* DSM 15163, *Meth. woesei* DSM 11979, *Meth. thaueri* DSM 11995, *Meth. millerae* DSM 16643, *Meth. oralis* DSM 7256, *Meth. olleyae* DSM 16632, *Meth. wolinii* DSM 11976, *Meth. arboriphilicus* DSM 1125, and *Meth. gottschalkii* DSM 11977). Also, the primer and probe sets were evaluated against the gDNA from 18 bacteria (*Aeromonas hydrophila* ATCC 7966, *Acinetobacter baumannii* ATCC 19606, *Bacteroides fragilis* ATCC 25285, *Burkholderia cepacia* ATCC 25416, *Citrobacter freundii* ATCC 8090, *Cronobacter sakazakii* ATCC 29544, *Enterobacter cloacae* ATCC 13047, *Enterococcus faecalis* ATCC 19433, *E. coli* ATCC 23229, *Klebsiella pneumoniae* ATCC BAA-1706, *Micrococcus luteus* CCT 2688, *Neisseria gonorrhoeae* IAL1894 (WHO-D), *Pantoea agglomerans* ATCC 33243, *Proteus mirabilis* ATCC 29906, *Pseudomonas aeruginosa* ATCC 27853, *Serratia marcescens* ATCC 13880, *Staphylococcus aureus* ATCC 12600, *Vibrio cholerae* ATCC 14035), and from three archaea, *Haloferax volcanii* DSM 3757, *Halococcus morrhuae* DSM 1307, *Haloarcula marismortui* DSM 3752, to check cross-reactivity between microorganisms. We also added control samples with known sewage input from two hospital wastewater treatment plants and an animal wastewater plant without human fecal matter. Additionally, the sets were checked against human, horse, swine, sheep, chicken, and bovine fecal samples.

qPCR analysis of MST markers

A 12-point, 10-fold serial dilution of gDNA *Meth. smithii, Meth. ruminantium, Meth. gottschalkii* and swine-specific *Bacteroidales* was run in triplicate with the initial concentrations of 1.8×10^{12}, 3.4×10^{12}, 2.2×10^{11}, and 1.0×10^{12} copies·μL^{-1} of the target gene, respectively. The gDNA stock concentration and the copy number was calculated according to Oliveira *et al.* (2016). The lowest number of gene copies that was detected consistently in the standard curves was considered the qPCR assay lower limit of quantification (qPCR ALLOQ).

To check for the presence of inhibitors in the samples, the commercially available TaqMan® Exogenous Internal Positive Control (IPC) Reagent (Thermo Fisher Scientific, Foster City, CA, USA) was adopted. The degree of PCR inhibition and correction of the values found were estimated across the difference between the CT average of the control and water samples (Rao *et al.* 2013; Oliveira *et al.* 2016).

The qPCR assays were optimized separately for each target and the reactions were performed in 20 μL containing 1X QuantiNova Probe MasterMix (Qiagen, Hilden, Germany), 0.25 μM of each primer (0.5 μM bovine), 0.2 μM (0.25 μM bovine) probe (Integrated DNA Technologies, Coralville, IA, USA), and 20 ng of sample DNA. It is remarkable that a single cycling condition was established for four markers aiming to decrease the analysis time. All samples were amplified in triplicate with a standard curve that also served as positive controls under the following conditions: 5 min at 95 °C, 40 cycles of 20 seconds at 95 °C, and 1 min at 58 °C. Amplification and fluorescence detection were performed in the real-time thermocycler Rotor-Gene® Q 5plex HRM platform (Qiagen, Hilden, Germany).

To confirm the identity and specificity of the amplified sequences, the qPCR fragments were purified using the QIAquick® PCR Purification kit (Qiagen GmgH, Hilden, Germany) and sequenced as described above. Chromatograms were converted to the FASTA format through Sequencher 5.0 software (Gene Codes Corporation, Ann Arbor, MI, USA). Nucleotide similarity searches were carried out online with BLASTn (http://www.ncbi.nlm.nih.gov/BLAST/) against GenBank (NCBI).

RESULTS

Physical chemical parameters and FIB enumeration

The pH values ranged between 4.1 and 12.2, and dissolved oxygen (DO) was higher at the mouth of the São João River and lower in the Queimados River. The Bacaxá River showed a high turbidity value (202 NTU) due to suspended matter in the shallow river, and Queimados River presented high conductivity levels that are directly proportional to the ionization of substances dissolved in the water. Out of the 64 samples, 29.7% (19/64) had DO levels below those recommended by the Brazilian water quality standards (Table 2).

In relation to pH values, Juturnaíba Dam ($p = 0.04102$) and Bacaxá ($p = 0.003324$) and São João ($p = 0.04196$) rivers presented significant variation among the collections performed in 2013 and 2014. In Guandu watershed, Santa Cecília Dam ($p = 0.05334$), Piraí River ($p = 0.01673$), Ribeirão das Lajes trough ($p = 0.006785$), Guandu River ($p = 0.03859$), Poços River ($p = 0.03074$), Queimados River ($p = 0.04675$), Guandu Lagoon ($p = 0.000466$), and Guandu Dam ($p = 0.00808$) presented significant variation between the analyzed samples. Meanwhile, the conductivity showed significant variations between the four collections in Bacaxá River ($p < 2.2 \times 10^{-16}$) and Juturnaíba Dam ($p < 2.2 \times 10^{-16}$) (São João Watershed). The same was observed in four (Ribeirão das Lajes trough ($p = 0.001096$), Macacos River ($p = 0.03746$), Santana River ($p = 0.002342$), and Guandu Lagoon ($p = 0.02493$)) of ten Guandu watershed rivers. Regarding the turbidity result, 84% (5/6) (Bacaxá River ($p = 0.02452$), Capivari River ($p = 0.006612$), Capivari Railway ($p = 0.02441$), Juturnaíba Dam ($p = 0.03249$), and São João River ($p = 0.03974$)) of São João watershed and 30% (3/10) (Ribeirão das Lajes trough ($p = 0.0009445$), Santana River ($p = 0.04572$) and Guandu River ($p < 2.2 \times 10^{-16}$)) of Guandu Watershed rivers varied significantly between the samples. The dissolved oxygen values of São João River mouth ($p = 0.01575$), Ribeirão das Lajes trough ($p = 0.001631$), Santana River ($p = 0.02875$), and Guandu Lagoon ($p = 0.001459$) showed significant variations between the samples. Only Ribeirão das Lajes trough presented a significant variation ($p = 0.037$) of temperature parameter (Table 3).

The total coliform concentrations ranged from 6 to >24,196 MPN/100 mL and it is worth noting that 48% (19/40) and 83% (20/24) of Guandu and São João samples showed values >10,000 MPN/100 mL, respectively. *E. coli* concentrations exceeded Brazilian regulatory guidelines (1,000 MPN/100 mL) in 38% of both watersheds, Guandu (15/40) and São João (9/24), with levels ranging from 1,120 to >24,196 MPN/100 mL. The Queimados and Macacos Rivers (Guandu watershed) presented the highest FIB concentrations among all samples. Also, 62.5% (40/64) of the water samples showed *E. coli* levels below the guideline limits (Figure 2).

Quantification of fecal contamination by qPCR

The qPCR assay lower limit of quantification of the *nifH* gene of *Meth. smithii* and *Meth. ruminantium* were 1.5×10^3 and 4.0×10^2 copies·L^{-1}, respectively. Meanwhile,

Table 2 | Physicochemical parameters of water samples from São João and Guandu watersheds

		pH	Conductivity (μS/cm)	Turbidity (NTU)	DO (mg/L)	Temperature (°C)	Salinity (‰)
Standard CONAMA 357/05 class II fresh waters		**6.0 to 9.0**	**–**	**<100**	**>5.0**	**–**	**≤0.5**
Bacaxá River	November/2013	6.8	0.1	202*	4.4*	26	0.0
	May/2014	6.9	0.1	35	5.4	30	0.0
	November/2014	6.9	0.1	3	5.1	26	0.0
	May/2015	8.5	0.1	4	5.4	30	0.0
Capivari River	November/2013	6.5	0.0	66	3.3*	30	0.0
	May/2014	7.0	0.0	10	5.4	26	0.0
	November/2014	6.4	0.1	15	4.7*	25	0.0
	May/2015	4.8*	0.1	14	5.0	31	0.0
Capivari Railway	November/2013	6.3	0.0	79	3.1*	31	0.0
	May/2014	7.3	0.1	12	5.7	28	0.0
	November/2014	6.8	0.3	13	4.2*	22	0.0
	May/2015	7.6	0.8	23	5.1	12	0.0
Juturnaíba Dam	November/2013	6.9	0.1	1	5.2	33	0.0
	May/2014	7.3	0.1	3	5.8	28	0.0
	November/2014	7.3	0.1	1	4.8*	25	0.0
	May/2015	9.0	0.1	12	5.3	30	0.0
São João River	November/2013	7.7	0.3	78	5.4	27	0.0
	May/2014	7.6	0.3	20	5.2	25	0.0
	November/2014	7.3	0.0	5	5.6	25	0.0
	May/2015	5.9*	0.0	6	5.8	29	0.0
Santa Cecília Dam	November/2013	6.6	93.0	3	7.1	20	0.0
	May/2014	7.2	122.0	4	8.0	24	0.0
	November/2014	6.6	93.0	3	7.1	20	0.0
	May/2015	9.2*	107.0	0	5.2	16	0.0
Piraí River	November/2013	6.4	91.0	3	4.8*	21	0.0
	May/2014	7.0	124.0	3	7.5	24	0.0
	November/2014	6.9	116.0	4	5.6	21	0.0
	May/2015	10.8*	0.1	1	4.6*	23	0.0
Ribeirão das Lajes trough	November/2013	6.4	33.0	2	8.7	22	0.0
	May/2014	6.8	38.0	3	8.8	24	0.0
	November/2014	6.4	33.0	2	8.7	22	0.0
	May/2015	11.1*	177.0	33	6.4	13	0.0
Macacos River	November/2013	6.7	322.0	7	2.4*	23	0.0
	May/2014	7.4	394.0	10	4.4*	26	0.0
	November/2014	6.7	322.0	7	2.4*	23	0.0
	May/2015	9.2*	0.2	4	5.1	22	0.0
Santana River	November/2013	6.7	60.0	6	9.3	23	0.0
	May/2014	7.5	57.0	7	9.9	27	0.0
	November/2014	6.7	60.0	6	9.3	23	0.0
	May/2015	8.4	0.6	2	6.2	17	0.0
Guandu River	November/2013	6.6	89.0	3	8.8	22	0.0
	May/2014	7.1	121.0	3	7.9	24	0.0
	November/2014	6.5	0.1	3	5.4	23	0.0
	May/2015	4.1*	0.1	6	5.9	22	0.0
Poços River	November/2013	7.0	586.0	20	2.3*	22	0.0
	May/2014	7.3	321.0	10	4.2*	27	0.0
	November/2014	6.6	89.0	3	8,8	22	0.0
	May/2015	10.2*	113.0	3	6.0	14	0.0

(*continued*)

Table 2 | Continued

		pH	Conductivity (μS/cm)	Turbidity (NTU)	DO (mg/L)	Temperature (°C)	Salinity (‰)
Queimados River	November/2013	6.8	238.0	13	0*	22	0.0
	May/2014	7.4	649.0	49	0*	27	0.0
	November/2014	7.2	446.0	33	0.7*	20	0.0
	May/2015	9.4*	0.4	15	2.5*	22	0.1
Guandu Lagoon	November/2013	7.8	335.0	25	8.3	21	0.0
	May/2014	7.9	395.0	19	8.2	25	0.0
	November/2014	7.8	335.0	25	8.3	21	0.0
	May/2015	12.2*	0.1	3	5.8	11	0.0
Guandu Dam	November/2013	7.0	84.0	4	8.2	22	0.0
	May/2014	7.3	123.0	3	7.7	25	0.0
	November/2014	7.2	0.1	3	4.1*	24	0.0
	May/2015	4.4*	0.1	1	5.2	24	0.0
Standard CONAMA 357/05 class II brackish waters		**6.5 to 8.5**	–	**<100**	**>5.0**	–	**≥0.5 to 30**
São João River mouth	November/2013	8.7	18.9	15	11.1	23	1.1
	May/2014	7.5	43.4	5	5.3	27	2.9
	November/2014	7.7	36.6	1	4.8*	25	2.3
	May/2015	6.0	43.9	2	5.7	28	2.8

*Above detection limit.

the *mcr*A gene of *Meth. gottschalkii* showed values of 2.3×10^3 copies·L^{-1} while the 16S rRNA gene of swine-associated *Bacteroidales* was quantified up to 1.2×10^3 copies·L^{-1}. The correlation coefficient for all qPCR assays was always greater than 0.99, with efficiencies between 98% and 109%. The slope of the curves ranged from −3.19 to

Table 3 | *P*-value of physicochemical parameters of water samples from São João and Guandu watersheds

	***p*-value**				
	pH	**Conductivity**	**Turbidity**	**DO**	**Temperature**
Bacaxá River	0.003324	$<2.2 \times 10^{-16}$	0.02452	0.092	0.8453
Capivari River	0.07726	0.8453	0.006612	0.1012	0.6413
Capivari Railway	0.367	0.1268	0.02441	0.3223	0.2104
Juturnaíba Dam	0.04102	$<2.2 \times 10^{-16}$	0.03249	0.2981	0.4158
São João River	0.04196	0.8453	0.03974	0.4508	0.2592
São João River mouth	0.2357	0.08292	0.0719	0.01575	0.3464
Santa Cecília Dam	0.05334	0.242	0.0755	0.134	0.367
Piraí River	0.01673	0.06202	0.1457	0.1097	0.4444
Ribeirão das Lajes trough	0.006785	0.001096	0.0009445	0.001631	0.037
Macacos River	0.07974	0.03746	0.367	0.5335	0.0755
Santana River	0.2301	0.002342	0.04572	0.02875	0.2214
Guandu River	0.03859	0.5269	$<2.2 \times 10^{-16}$	0.5135	0.2592
Poços River	0.03074	0.2155	0.1761	0.3247	0.2024
Queimados River	0.04675	0.4046	0.3059	0.07974	0.1023
Guandu Lagoon	0.000466	0.02493	0.0755	0.001459	0.1023
Guandu Dam	0.00808	0.4635	0.1457	0.5095	0.1457

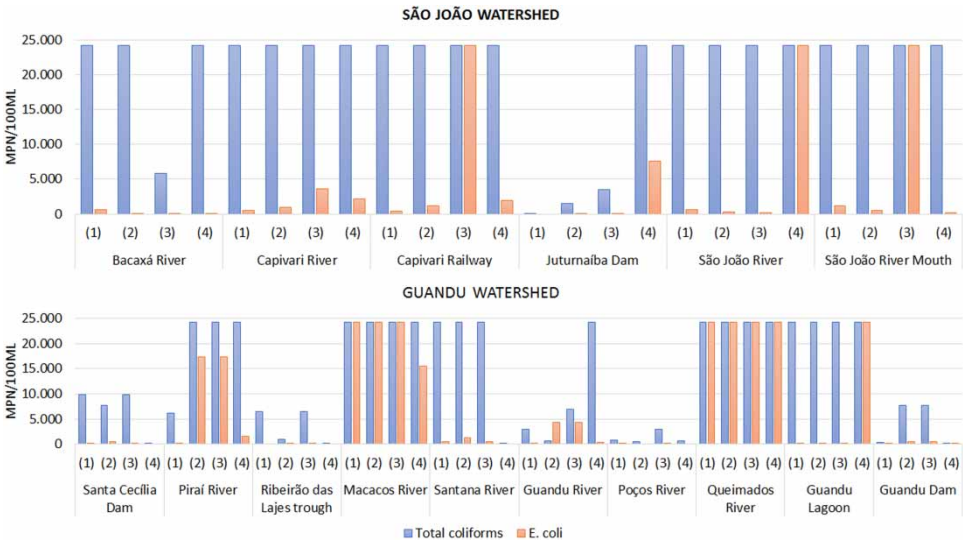

Figure 2 | Enumeration of total coliform and *E. coli*: (1) November/2013; (2) May/2014; (3) November/2014; (4) May/2015) from two watersheds studied.

−3.37. As expected, the IPC indicated inhibition for most water samples; thus, the values were adjusted by a correction factor to calculate the real number of copies per liter of samples. Also, as expected, there was no cross-reaction in hospital and animal effluent samples (Figure 3).

The results showed high concentrations of fecal contamination in the analyzed samples, suggesting the presence of continuous contamination of human, swine, and bovine feces (Figure 4). The *nifH* gene of *Meth. smithii* from human feces showed values of approximately 2.0×10^{14} copies·L^{-1}, and all water samples for this marker oscillated from 8.6×10^{2} to 6.3×10^{10} copies·L^{-1}, at Guandu River and Queimados River, respectively. Meanwhile, the *Meth. ruminantium nifH* gene exhibited 8.7×10^{12} copies·L^{-1} of bovine feces and in 92% (59/64) of water samples, ranging from 2.5×10^{2} copies·L^{-1} in the Guandu Dam to 3.3×10^{12} copies·L^{-1} in Santana River. Swine feces presented 3.1×10^{11} copies·L^{-1} of the swine-specific *Bacteroidales* 16S rRNA gene. This bacterial marker was quantified in 89% (57/64) of the water samples, ranging from 1.5×10^{3} to 4.8×10^{12} copies·L^{-1} in the Queimados and Poços Rivers, respectively. The *mcrA* gene

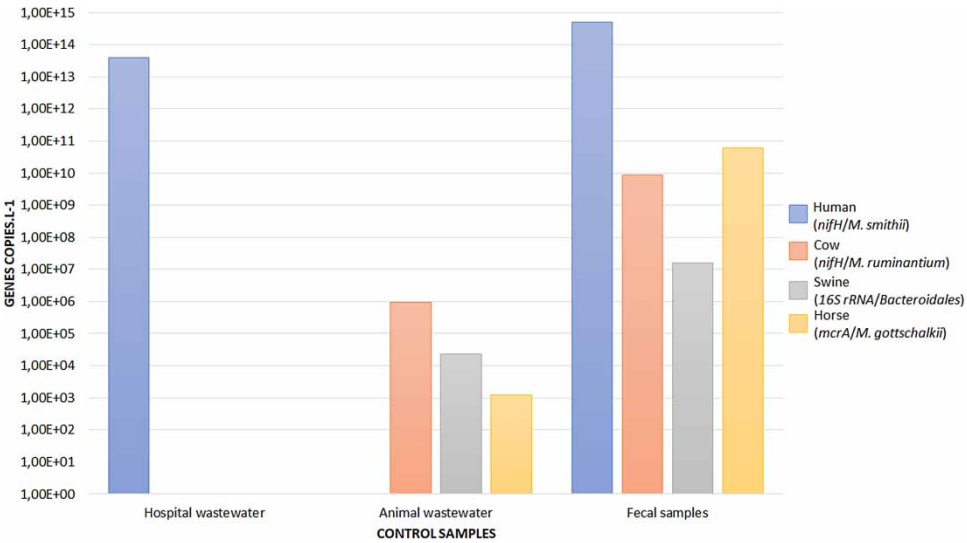

Figure 3 | Quantification of human, cow, swine, and horse specific markers of fecal contamination in control samples.

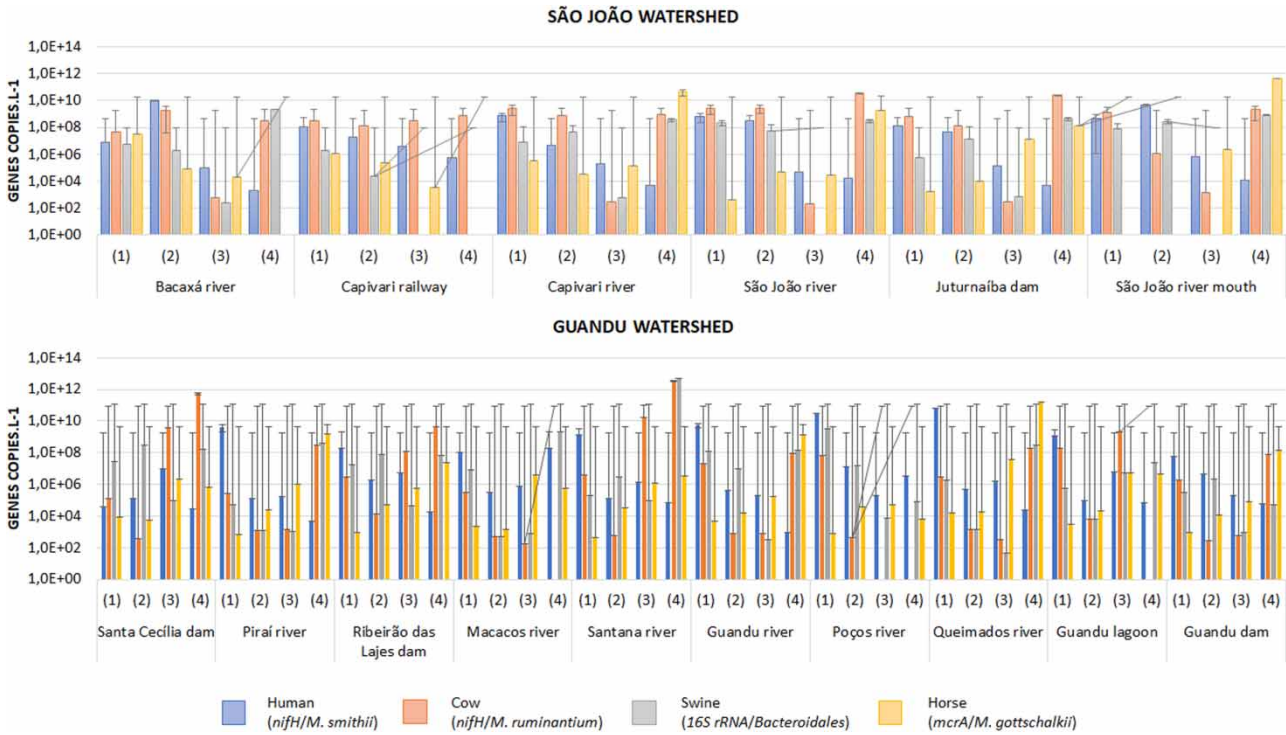

Figure 4 | Quantification of human, cow, swine, and horse specific markers of fecal contamination in water samples: (1) November/2013; (2) May/2014; (3) November/2014; (4) May/2015) from two watersheds studied.

of *Meth. gottschalkii*, the horse fecal contamination marker, resulted in 1.6×10^{10} copies·L^{-1} in horse feces and was detected in 81% (52/64) of the samples, ranging from 1.4×10^{3} copies·L^{-1} in Macacos River to 4.6×10^{11} copies·L^{-1} in the São João River mouth (Figure 4).

DISCUSSION

Intensive human activities followed by uncontrolled disposal of wastes are diminishing available water supplies through overuse as well as fecal and chemical pollution. Consequently, studies have justified the concerns with these environments, considering the often unsustainable use of aquatic ecosystems (Behera *et al.* 2011; Lawford *et al.* 2013; Grizzetti *et al.* 2016), increased pollution (Dudgeon *et al.* 2006; Vörösmarty *et al.* 2010; Alho *et al.* 2015; Holden *et al.* 2016), and inadequate management of water distribution systems.

This study aimed to identify the host-specific fecal contamination in the Guandu and São João watersheds to reveal the pollutants in these ecosystems and predict their possible negative impacts on human health. Overall, there were no significant differences concerning physicochemical parameters between samples from different seasons. However, away from inhabited areas and known sewage discharges, the two sampled points of the Capivari River (São João watershed) as well as several other points showed low levels of dissolved oxygen. According to Igbinosa & Okoh (2009), concentrations of oxygen below 5 mg·L^{-1} can affect aquatic life, since in unpolluted environments this concentration normally ranges between 8 and 10 mg·L^{-1}. Our data could indicate not-point releases of pollutants that compromise the quality of water intended for drinking water supply since the impairment of these watersheds is alarming, which makes treatment more expensive and unlikely.

The sudden pH changes demonstrated, mainly in Guandu watershed, may be associated with high pollution levels in the rivers that make up this watershed. Pollution is a common cause that can raise or lower the pH depending on the waste involved, for example, chemicals from agricultural runoff, effluent discharge, or industrial runoff. The discharge

of wastewater containing detergents and soap-based products can make the pH rise and the water very basic (Yang *et al.* 2008). The presence of high electrical conductivity (EC) values in Guandu watershed may be associated, mainly with the high discharges of fresh industrial effluents containing, among others, compounds of chloride, phosphate, and nitrate (Şener *et al.* 2017). The same did not occur in the São João rivers, and it must be kept in mind that they are located in rural areas, free from industrial activities.

Our data revealed a weak correlation between *E. coli* enumeration and archaeal and bacterial MST markers. The 40 samples within acceptable limits of *E. coli* revealed at least three host-specific fecal markers. On the other hand, the other 24 samples with *E. coli* levels above those recommended revealed the presence of all fecal pollution markers analyzed. Similar results were observed in a study by Johnston *et al.* (2010) where there was little or no correlation between the detection of human fecal contamination marker genes and *E. coli* and *Enterococcus faecalis* enumeration. Indeed, the comparison of *E. coli*, total coliform and enterococci by culture-based and qPCR methodology showed disparity between these two approaches (Ervin *et al.* 2013; Wuertz *et al.* 2015; Rodrigues & Cunha 2017). In addition, high total coliforms and *E. coli* levels in water bodies are not associated with pathogens and have poor predictive relationships to human health risks. Therefore, the absence of these indicators in water samples cannot guarantee the absence of pathogens (Horman *et al.* 2004; Boehm *et al.* 2009; José Figueras & Borrego 2010; Yousefi *et al.* 2018). As expected, these bioindicators were found in most samples analyzed in this study. However, it is noteworthy that Macacos and Queimados rivers (Guandu watershed) revealed values 20-fold higher than the limits permitted by Brazilian water quality guidelines, and certainly due to a large discharge of domestic, hospital, and industrial wastewater in these waters.

The sensitivity and specificity of the *nifH* gene (*Meth. smithii*) as a marker was investigated against fecal samples from several animal species, including environmental samples, demonstrating specificity of 96% and 81% of sensitivity against human sewage (Ahmed *et al.* 2012). In addition, it is worth noting that an optimized protocol for DNA extraction and a PCR protocol for the specific detection of *Meth. smithii* in stool samples allowed the detection of the *rpoB* gene in the feces of almost all individuals (95.5%) (Dridi *et al.* 2009). On the other hand, in a previous study, we also evaluated the specificity and sensitivity of the *nifH* and 16S rRNA genes as fecal markers against water samples and fecal matter from six animal species. The specificity and the sensitivity were 100% and 91% for the human and bovine markers, respectively (Bianco *et al.* 2015). The prevalence of these MST markers was relatively greater in comparison with the established *Bacteroidales* (16S rRNA) markers in water samples.

In the present study, we demonstrated high copy numbers of the human- and bovine-associated *nifH* gene markers in almost all samples, regardless of the *E. coli* levels, reaffirming the weak correlation between conventional and host-specific markers adopted. Interestingly, human-associated *nifH* gene detected in treated sewage showed low correlation with cultivable *Enterococcus* but a high correlation with norovirus, *Cryptosporidium* spp., and *Giardia lamblia* (Rosario *et al.* 2009). Furthermore, high concentration of *nifH* gene could indicate recent discharge of human fecal matter in the study sites. This issue requires the attention of local authorities since, according to the Rio de Janeiro State Environmental Institute (INEA), the Pontal beach that receives the waters of the São João River was inappropriate for bathing. This area has been suffering from irregular sewage dumping due to the lack of sanitation in this region, which makes it an even more serious problem and a health risk in relation to the water quality of this beach.

Our qPCR results also demonstrated swine fecal contamination in 89% of water samples by *Bacteroidales*-specific 16S rRNA gene, revealing high concentrations of swine fecal matter, probably from swine breeding and sewage discharge in aquatic environments by the local population. The equine fecal pollution marker, *mcrA* gene, was detected at lower levels compared to the human, bovine, and swine markers. The application of *mcrA* gene in methanogen searching of *Methanobacteriales* order showed remarkably similar phylogenetics of *mcrA* and 16S rRNA genes, which validated the application of this genetic marker to reveal methanogenic archaeal organisms (Luton *et al.* 2002; Evans *et al.* 2019). Additionally, Ufnar *et al.* (2007) demonstrated the potential of the *mcrA* gene in the exploration of host-specific methanogens for microbial source tracking, as well as in establishing a swine-specific marker for fecal pollution.

Several microorganisms have been suggested as alternative bioindicators of fecal contamination in water systems, but the application of archaeal and bacterial markers together by qPCR assay is relatively recent. Aquatic environments contaminated by animal feces pose as much risk to human health as those contaminated only by human feces. It is remarkable that pathogen presence in feces is not exclusive to ill humans and animals as healthy animals' feces could also be reservoirs of pathogens (Zheng & Shen 2018). For example, cattle are a reservoir for enteropathogenic *E. coli* (EPEC) (Vasco *et al.* 2016) while *Campylobacter coli* (Thépault *et al.* 2018) is prevalent in swine waste. In addition, human infections caused by *Trichinella spirallis* and *Salmonella enterica* have been associated with equine feces. In addition to fecal bovine, swine and equine contamination, human feces is one of the main carriers of pathogens such as *Bacteroides* spp., *Salmonella* spp., *Shigella* spp., and *Campylobacter* spp. It is noteworthy that *Campylobacter* spp., non-typhoidal *Salmonella* (NTS), *Cryptosporidium* spp., and *Toxoplasma gondii* were considered the pathogens of highest concern which may substantially contribute to the global burden of disease in humans due to their spread in animal feces (Delahoy *et al.* 2018).

Thus, determining the source of fecal contamination may predict possible human health risks. Although there are studies available on this subject, there is a need for more transdisciplinary studies encompassing the knowledge gained about the presence of fecal contaminants that provide a comprehensive synopsis for a better understanding of this issue.

CONCLUSION

The molecular marker genes proposed for qPCR analysis in our study were sensitive and specific enough to detect host-specific fecal pollution in all samples regardless of *E. coli* levels, reaffirming the low correlation among them and supporting their use in water quality monitoring programs.

The high concentrations of human and animal fecal contamination in water intended for treatment and public supply revealed in this study highlights the need for improvements in basic sanitation infrastructures along these watersheds.

Finally, we conclude that these two watersheds directly threatened by disorganized urbanization, industrialization, and continuous pollution could lead to the collapse of the water supply system.

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