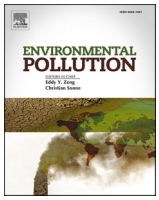
Environmental Pollution 363 (2024) 125089

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol



Environmental surveillance of *Cryptosporidium* and *Giardia* in surface supply water and treated sewage intended for reuse from an urban area in Brazil☆

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ARTICLE INFO

Keywords:
Waterborne protozoan
Water supply
Reuse water
Environmental monitoring
qPCR
Public health

ABSTRACT

Environmental monitoring of protozoa, with the potential to trigger diseases, is essential for decision-making by managing authorities and for the control of water surveillance. This study aimed to detect and quantify *Cryptosporidium* oocysts and *Giardia* cysts in surface water for drinking water supply and treated sewage for reuse in the city of São Paulo. Samples collected bimonthly for one year were concentrated using the USEPA 1623.1 and 1693 methods for surface water and treated effluents, respectively. Immunofluorescence and nucleic acid amplification techniques were used to detect and quantify (oo)cysts. The cloning technique followed by sequencing and phylogenetic analyses were performed to characterize species and genotypes. The immunofluorescence detected *Cryptosporidium* spp. and *Giardia* spp. in 69.2% (9/13) and 100% (13/13) of the surface water samples (0.1–41 oocysts/L and 7.2–354 cysts/L, respectively). In the reuse samples, 85.7% (12/14) were positive for both protozoa and the concentrations varied from 0.4 to 100.6 oocysts/L and 1.2 and 93.5 cysts/L. qPCR assays showed that 100% of surface water (0.1–14.6 oocysts/L and 0.3–639.8 cysts/L) and reused samples (0.1–26.6 oocysts/L and 0.3–92.5 cysts/L) were positive for both protozoa. Species *C. parvum*, *C. hominis,* and *C. muris* were identified using the *18S rRNA* gene, demonstrating anthroponotic and zoonotic species in the samples. Multilocus *SSU rRNA*analyses of the *SSU rRNA*, *tpi*, and *gdh* genes from *Giardia intestinalis* identified the AII, BII, and BIV assemblages, revealing that contamination in the different matrices comes from human isolates. The study showed the circulation of these protozoa in the São Paulo city area and the impairment of surface water supply in metropolitan regions impacted by the discharge of untreated or inadequately treated sewage regarding the removal of protozoa, emphasizing the need to implement policies for water safety, to prevent the spread of these protozoa in the population.

1. Introduction

The contamination of water intended for public supply by biological agents poses risks to human health, especially regarding waterborne infectious diseases (WHO, 2022). Despite technological advances for the pathogenic agents removal from water catchment, their presence in these matrices constitutes a challenge for companies producing drinking water in developing countries. In this regard, monitoring the water-producing system and reducing contamination at the catchment, that is, protection of water sources, are still the most effective and least expensive ways to protect water quality (Araújo et al., 2018).

Cryptosporidium and *Giardia* are currently the main protozoa

☆ This paper has been recommended for acceptance by Dr. Sarah Harmon.

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https://doi.org/10.1016/j.envpol.2024.125089
Received 14 June 2024; Received in revised form 1 October 2024; Accepted 5 October 2024
Available online 9 October 2024

detected in waterborne outbreaks in different continents and present a high risk to the population's health due to their ability to tolerate changes in environmental conditions and resist conventional treatments applied to drinking water (Baldursson and Karanis, 2011; Benedict et al., 2017; Rosado-García et al., 2017; Efstratiou et al., 2017a; Hlavsa et al., 2021; Bourli et al., 2023). Both giardiasis and cryptosporidiosis can be zoonotic or anthropogenic, which increases their transmission capacity, primarily through the fecal-oral route due to exposure to contaminated food and water. The clinical picture can vary from mild to severe depending on the nutritional and immunological status of the affected individuals. In the case of cryptosporidiosis, there is evidence that symptoms vary according to the infection site in the host and the infecting species (Chalmers and Davies, 2010).

Symptomatic giardiasis can result in nausea, diarrhea, anorexia, malaise, and occasionally low-grade fever or chills (Taran-Benshoshan et al., 2015). *G. lamblia* is the third most common agent responsible for diarrheal diseases in the world, with more than 300 million cases per year, preceded only by rotavirus and *Cryptosporidium parvum/hominis* among vulnerable individuals (Cernikova et al., 2018).

A study of the global distribution of waterborne outbreaks by protozoa revealed that *Cryptosporidium* spp. and *Giardia* spp. were the most reported etiological agents in outbreaks until 2010 (Baldursson and Karanis, 2011). Between 2011 and 2016, 381 waterborne outbreaks were reported, and *Cryptosporidium* was the most recurrent, contributing 63% (239) of the episodes, followed by *Giardia* with 37% (142) (Efstratiou et al., 2017b). An update of global waterborne protozoan outbreaks, published by Bourli et al. (2023), revealed that between 2017 and 2022 the percentage of waterborne *Cryptosporidium* outbreaks increased to 77.4% (322/416), while those of *Giardia* decreased significantly (17.1%).

In Brazil, as has been observed in different regions of the world, these protozoa have already been widely detected in both clinical and environmental samples (Branco et al., 2012; Breternitz et al., 2020; Coelho et al., 2017; Araújo et al., 2018; Fantinatti et al., 2020; Fernandes et al., 2011; Franco et al., 2016; Leal et al., 2018; Paz e Silva et al., 2014; Sato et al., 2013; Zini et al., 2021).

A systematic review of giardiasis in Brazil (Coelho et al., 2017), evaluating studies published from 1995 to 2015, showed a maximum prevalence of the disease in humans of 69.9% in the state of São Paulo in 1998, and highlighted the difficulty of making a temporal comparison due to the lack of studies over the years. Data from the Hospital Information System of the Federal Government's Unified Health System (SUS) show that in the period from 2017 to 2023, 39 hospitalizations due to giardiasis were recorded, and there are no records of cryptosporidiosis (DATASUS, 2024). There is a gap in the reporting cases in the country since there is no established epidemiological surveillance for these protozoa.

Despite advances in basic sanitation in Brazil, the population without access to these services is still high. According to the National Sanitation Information System (SNIS), almost 100 million Brazilians do not have access to basic services and in 2019, more than 273,000 hospitalizations due to waterborne diseases were reported in the country (BRASIL, 2023). Thus, disorderly urban growth and the release of untreated or inadequately treated domestic and industrial sewage into rivers, streams, and lakes contribute to the degradation of surface water resources and the proliferation of diseases, impairing the quality of life of the population (BRASIL, 2023).

Given this scenario, the procedures for controlling and monitoring the quality of potable water and its standards are stated in the National Ordinance GM//MS N° 888, revised in May 2021. The control of water safety in Brazil is performed according to compliance with standards for *Escherichia coli* (BRASIL, 2021). This Ordinance also provides the control and monitoring procedures for the removal of pathogens, including the search of *Cryptosporidium* oocysts and *Giardia* cysts at the water intake points of treatment plants that have high fecal contamination (>1000 *E. coli*/100 mL). However, there is no standardized methodology for this requirement and many production systems do not meet the requirement. Also, in Brazil, there are specific regulations for the protection of water sources, however, in large urban centers, urban sprawl impacts the water quality of the catchment areas.

In addition to the water crises caused by human actions, the current extreme weather events are one of the greatest challenges faced by humanity in this century, as they negatively impact the quality and availability of drinking water for the population (Masangkay et al., 2020). In this scenario, the increasing demand for water has encouraged the practice of water reuse for sustainable supply, also to avoid the shortage of drinking water, especially in developing countries where the lack of resources and regulation is a concern (Rosado-García et al., 2017).

In Brazil, regulations that establish quality standards for water reuse are scarce. Some guidelines and general criteria for the practice of direct reuse of water are contained in Resolution N° 54 of November 28, 2005, of the National Water Resources Council (CONARH) (BRASIL, 2005). However, the State of São Paulo, in 2017, implemented a Joint Resolution that imposes restrictions based on two main categories, severe and moderate, for the direct non-potable reuse of water for urban purposes, from Sanitary Sewage Treatment Stations (SES/SMA/SSRH, 2020). The concern with the implementation of criteria for the reuse of wastewater, which focuses on sustainable public health and well-being, is in line with the interests of the Sustainable Development Goals (SDGs), which include the commitment of governments to reduce the number of deaths and diseases due to air, water and soil contamination (CETESB, 2020).

Nevertheless, in the Metropolitan Region of São Paulo, the recovered water generated in the Wastewater Treatment Plants (WWTPs) has been used only for urban purposes, such as landscape irrigation, street and vehicle washing, civil construction, among others (Prado et al., 2019). Razzolini et al. (2020) evaluated the occurrence of *Giardia* and *Cryptosporidium* in wastewater used for street washing in the city of São Paulo, revealing that the commitment of authorities to the microbial quality of reuse water is still lacking, which is one of the main challenges for its safe use.

In this sense, water recycling can provide stable and sustainable water resources for a variety of uses, however, the reuse of wastewater must be carried out carefully and responsibly due to the presence of pathogenic organisms that are difficult to remove and inactivate by conventional treatment methods (Drigo et al., 2021). Within this perspective, our study aimed to evaluate the occurrence and identify species of *Giardia* and *Cryptosporidium* in samples of surface and reuse water used for different purposes, in two drinking water treatment plants (DWTP) and two wastewater treatment plants (WWTP) located in the Metropolitan Region of São Paulo, highlighting the importance of implementing water safety plans and establishing quality criteria for reused water with a focus on microbial risk assessments to protect public health in our country.

2. Material and methods

2.1. Study area and sampling

The present study was carried out in the metropolitan region of São Paulo (MRSP), an urban area with approximately 20 million inhabitants and serious socioeconomic and environmental impacts (Marengo and Alves, 2015). The basic sanitation structure to serve this population includes eight DWTPs and five WWTPs, ensuring around 100% of the water supply, 81% of sewage collection, and 68% of sewage treatment (BRASIL, 2023).

Surface water sources - Two collection sites were selected within the Alto Tiete River Basin of the metropolitan region of São Paulo, one located at Cotia River (COTI 03900/Baixo Cotia DWTP), and the other at Ribeirão dos Cristais (CRIS 03400/Cajamar DWTP) (Fig. 1). These rivers are used for public water supply, recreation, and irrigation, and are

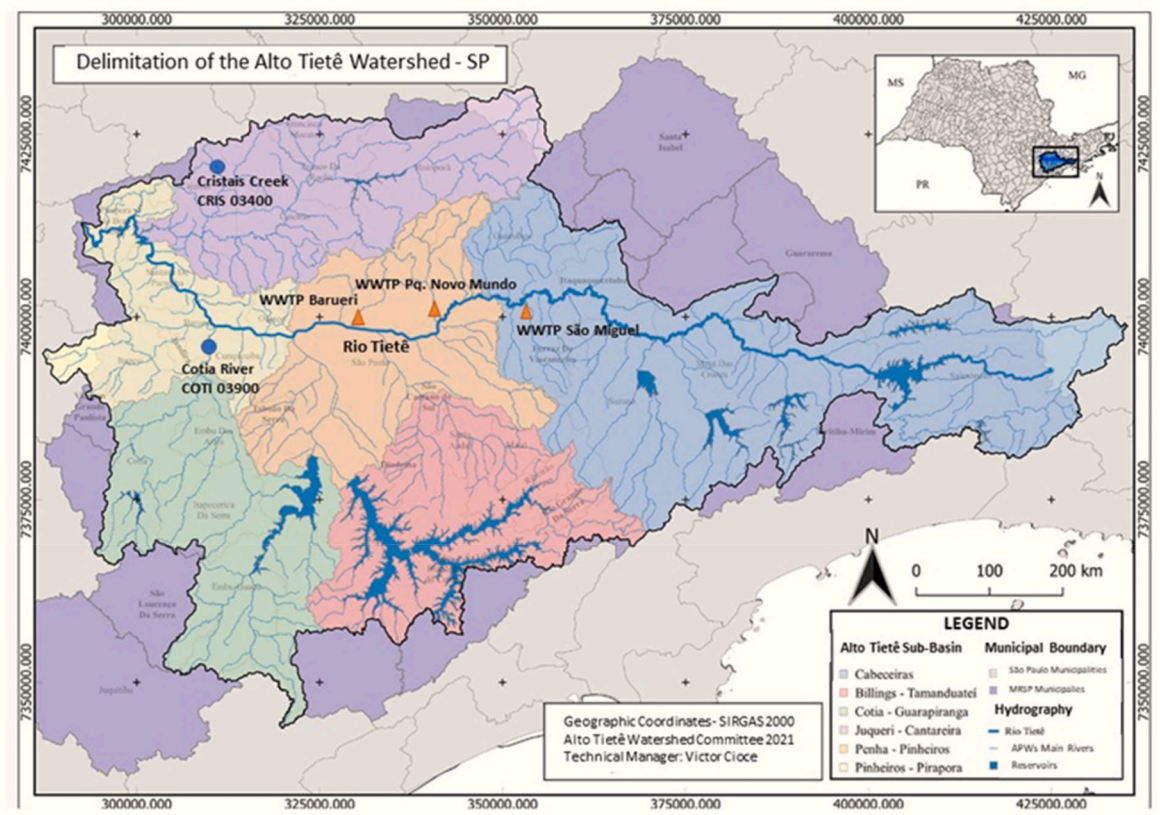

Fig. 1. Map of the metropolitan region of São Paulo (MRSP) with the location of sampled WWTPs and watersheds.

impacted by domestic waste from urban centers (CETESB, 2020).

Reuse water - Initially, two WWTPs were selected for reuse water monitoring (Barueri and São Miguel). However, in April 2019, the Barueri plant had to interrupt its reuse water production activities due to operational problems, requiring its replacement in this study by Parque Novo Mundo WWTP (Fig. 1). All WWTPs are located at MRSP serving

Table 1
Cryptosporidium and *Giardia* concentrations (IMS-FA) and bacteriological and turbidity results in surface water and reuse water samples.

Water source	Samples	Month/ year	Fecal indicator bacteria		Protozoa parasites		Turbidity (NTU)
			C. perfringens (CFU/100 mL)	*E. coli* (CFU/100 mL)	*Cryptosporidium* spp. (oocysts/L)	*Giardia* spp. (cysts/L)	
CRIS03400	1A	Nov/2018	ND	$2.6x10^4$	0.2	41.8	19.0
	2A	Feb/2019	140.0	$2.0x10^4$	0.6	87.7	55.3
	5A	Mar/2019	57.0	$2.4x10^4$	<0.1	7.2	38.6
	6A	May/2019	170.0	$6.3x10^3$	0.1	354.0	19.3
	9A	July/2019	130.0	$2.1x10^4$	<0.1	80.8	11.7
	10A	Sept/2019	200.0	$2.1x10^4$	0.9	6.4	40.4
	12A	Nov/2019	82.0	$5.4x10^4$	<0.1	130.5	13.3
COTI03900	3A	Feb/2019	$1.2x10^3$	$1.9x10^5$	3.83	154.0	11.1
	4A	Mar/2019	ND	$1.8x10^5$	12.7	48.8	26.2
	7A	May/2019	$2.4x10^3$	$3.9x10^5$	0.1	39.6	15.6
	8A	July/2019	$5.9x10^3$	$2.3x10^5$	<0.1	61.8	17.0
	11A	Sept/2019	420.0	$5.0x10^5$	41.2	198.8	19.4
	13A	Nov/2019	$5.5x10^3$	$6.3x10^5$	7.0	256.3	23.8
WWTP1	1R	Nov/2018	3.0	<1.0	1.6	4.0	0.1
	3R	Feb/2019	30.0	<1.0	100.6	93.5	29.6
	5R	Apr/2019	5.0	<1.0	1.2	11.6	4.6
WWTP2	2R	Nov/2018	<1.0	<1.0	<0.1	<0.1	4.0
	4R	Feb/2019	<1.0	<1.0	2.2	<0.1	1.4
	6R	Apr/2019	<1.0	<1.0	0.2	5.8	2.0
	7R	June/2019	21.0	<1.0	13.4	5.4	3.4
	10R	Aug/2019	59.0	<1.0	0.4	41.6	11.1
	12R	Sept/2019	112.0	<1.0	4.8	85.6	8.9
	14R	Dec/2019	1.0	1.0	4.0	20.8	3.7
WWTP3	8R	July/2019	<1.0	1.0	4.2	2.8	1.9
	9R	Aug/2019	<1.0	<1.0	1.8	9.6	1.2
	11R	Sept/2019	<1.0	<1.0	18.0	5.4	2.5
	13R	Dec/2019	72.0	<1.0	<0.1	12.0	1.1

CRIS03400 = Ribeirão do Cristais (10L); COTI03900 = Baixo Cotia (10L); WWTP = Wastewater Treatment Plant; WWTP1 =Barueri.
WWTP2= São Miguel; WWTP3 = Pq. Novo Mundo ND = Not detected; CFU = Colony-forming Unit; NTU = Nephelometric Turbidity Units.

approximately 8 million people. The treatments applied consist of primary treatment (sedimentation), secondary treatment (activated sludge), and tertiary treatment (filtration with sand-anthracite filter/reverse osmosis and chlorination). The tertiary final effluent is mainly used for urban purposes such as street washing, landscape irrigation, and civil construction (Prado et al., 2019). The characteristics of WWTPs are presented in Table S1 (Supplementary material).

Both surface and reuse water samples (30-L split into three 10-L sterile bottles) were collected at each sampling site bimonthly from November 2018 to November 2019, totaling 13 surface water and 14 reuse water samples (Table 1). Samples were kept at 4 °C for transportation and processed within 24 h, following the National Guide for the Collection and Preservation of Samples (CETESB, 2011) and Standard Methods for the Examination of Water and Wastewater (APHA, AWWA, WEF et al., 2017).

2.2. Physicochemical and microbiological evaluation

Physicochemical parameters were monitored to evaluate the interference of the abiotic parameters in the biological variables. Water and air temperature, as well as water pH, conductivity, and residual chlorine, were measured *in situ* with multiparameter probes according to the Standard Methods for the Examination of Water and Wastewater (SMWW), Sections 2550B, 4500H+ and 2510B APHA, AWWA, WEF et al., 2017). Total organic carbon (TOC) contents and turbidity were analyzed at the CETESB Chemistry Laboratory, also according to the SMWW, Sections 5310C and 2130, respectively (APHA, AWWA, WEF et al., 2017).

In addition to the concentrations of protozoan (oo)cysts, the indicators of fecal contamination *E. coli* and *Clostridium perfringens* were also quantified in surface and reuse water samples. The analyses of *E. coli* and *C. perfringens* were performed using the membrane filter technique according to the methodologies described in the SMWW (APHA, AWWA, WEF et al., 2017) and in the ICR Microbial Laboratory Manual (USEPA, 1996), respectively.

2.3. Giardia and Cryptosporidium analyses

2.3.1. Samples concentration and purification

Concentration and purification of (oo)cysts from surface and reuse water samples were performed according to Method 1623.1 (USEPA, 2012) and Method 1693 (USEPA, 2014), respectively. Briefly, individual 10-L volumes were combined, homogenized, and divided again into 10-L aliquots for filtration through Envirocheck® HV capsules (1 μm porosity) prepared according to the manufacturer's specifications (Pall corporation® USA).

The three eluates obtained for each sample were concentrated through centrifugation and the entire pellets obtained (≤0.5 ml) were purified by immunomagnetic separation (IMS) employing magnetic beads conjugated to anti-*Cryptosporidium* and anti-*Giardia* antibodies (Dynabeads™ GC-Combo, Applied Biosystems) and recovered by acid dissociation. The procedure adopted for reuse water was the same as Method 1623.1, except for the IMS step, in which an additional PBS buffer wash of the magnetized sample-antibody complex was included.

Two replicates were analyzed in the present study, one for quantifying (oo)cysts by immunofluorescence assay (FA), and the other for molecular procedures. In contrast, a third replicate was the object of viability and infectivity studies, which are not part of the scope of this article. Slides were analyzed immediately after concentration and genetic material was extracted and stored at −20 °C until PCR assays were performed. The flowchart containing the distribution of the samples is shown in Fig. S1 (Supplementary material).

2.3.2. Detection and quantification by direct immunofluorescence (IMS-FA)

The protozoan (oo)cysts obtained after the IMS process with acid dissociation (100 μL) were fixed on slides and stained with fluorescent antibody (Aqua-Glo™ G/C Direct FL or MeriFluor® *Cryptosporidium/Giardia*) and DAPI (4′,6- diamino-2-phenyl-indole, Sigma Aldrich®, USA) and was analyzed under an immunofluorescence microscope equipped with a Normaski-type differential and interferential phase contrast (Olympus) (Fig. S4 - Supplementary material). The initial precision and recovery (IPR) and the contaminated matrix recovery (MS) were determined and certified with reference material for surface and reuse water (ColorSeed™-BTF Bio, Australia) as recommended by USEPA (2014).

2.3.3. Molecular assays

2.3.3.1. DNA extraction. The concentrated eluate (100 μL) was extracted using the commercial DNeasy PowerSoil® kit (Qiagen, Germany), and processed according to the manufacturer's instructions. Briefly, a pretreatment of five cycles of heating at 95 °C for 5 min in a water bath and freezing for 5 min in dry ice was performed (Yu et al., 2009). The volume of 60 μL of buffer C1 was added directly to the eluate aliquot. DNA was purified using silica-gel columns, eluted in 100 μL of buffer C6, and stored at −20 °C.

2.3.3.2. Synthetic DNA curve standardization. Synthetic DNA fragments (gBlock™ Gene Fragment, IDT, Iowa, USA) were used to prepare standard curves. The 320-bp target region for *Cryptosporidium* was extracted from the *18S rRNA* gene of reference strain *C. parvum* Iowa II (NCBI: txid353152), and the 140-bp target region for *Giardia* was extracted from the *SSU rRNA* gene of reference strain *G. lamblia* H3 (NCBI: txid598745). Primers and probes used for amplification are in Table S2 (Supplementary material). Ten-fold serial dilutions of gBlock™ were used to construct the standard curves, reaching from 3.2×10^8 to 3.2×10^1 gene copies per μL (GC/μL) in each amplification cycle (Cq) for the *18S rRNA* gene of *C. parvum/C. hominis*, and 6.0×10^6 to 6.0×10^1 GC/μL for the *SSU rRNA* gene of *G. intestinalis* (Fig. S2 and Fig. S3 - Supplementary material). The standard curve was generated using linear regression of the relationship between quantitation cycle value and copy number.

2.3.3.3. qPCR reaction. Quantitative polymerase chain reactions (qPCRs) were performed using the StepOne Plus™ Real-Time PCR and the TaqMan-MGB probe systems (Applied Biosystems, Foster City, CA, USA). Amplification was performed in a total volume of 25 μL/reaction containing 12.5 μL of the TaqMan® Environmental Master Mix 2.0 kit (Applied Biosystems, Foster City, CA, USA) along with 5 μL of the template DNA and respective primers (20 pmol) and probe (150 pmol). Cycling conditions were 60 °C for 30 s for uracil-N-glycosylase activation, initial denaturation at 95 °C for 10 min and 40 cycles of 95 °C for 15 s followed by 60 °C for 1 min for annealing and extension. Quality controls included positive DNA samples obtained from purified suspensions purchased from Waterborne™ Inc., and non-template controls (NTC). Samples crossing the threshold line, presenting a characteristic sigmoid curve, and Cq values reaching the maximum of 38 (Cq ≤ 38) were considered positive. An internal positive control (IPC) (Applied Biosystems, USA) reaction was used to assess the presence of inhibitors in the samples.

The quantified values of gene copies/μL DNA (GC/μL) were converted into numbers of gene copies/L DNA (GC/L) according to Equation (1), considering that the total volume of the concentrated sample (100 μL) was subjected to DNA extraction. The conversion of gene copies/L DNA to (oo)cyst/L (Equation (2)) was determined to obtain comparable data to the USEPA Method (Mahmoudi et al., 2017; Araújo et al., 2018; Mthethwa et al., 2022; Hachimi et al., 2024). The number of genomic copies for the *18S rRNA* gene of *Cryptosporidium* (5 copies per genome) was calculated considering intact oocysts with 4 haploid sporozoites (Le Blancq et al., 1997; Abrahamsen et al., 2004) while the number of copies

for the *SSU rRNA* gene of *Giardia SSU rRNA* (120 copies per nuclei) was calculated considering intact mature cysts with 4 tetraploid nuclei (Adam, 2001; Bernander et al., 2001).

$$N^{\circ}\ GC \Big/ L = \frac{N^{\circ}\ GC/\mu L^{*}\ Extracted\ DNA(\mu L)}{Water\ sample\ (L)} \quad (1)$$

$$N^{\circ}\ (oo)cyst \Big/ L = \frac{N^{\circ}\ GC/L}{(oo)cyst\ N^{\circ}\ GC} \quad (2)$$

Where:

Extracted DNA = total elution volume of DNA (100 μL)

Water sample = total volume of water concentrate (10 L)

(oo)cyst N° GC = number of genomic copies per (oo)cyst (20 copies to *Cryptosporidium* and 480 copies to *Giardia*)

2.3.3.4. Evaluation of qPCR method performance. Initial precision and recovery (IPR) and matrix spiked recovery (MS) evaluations of quantitative PCR assays were performed by experimental seeding with known concentrations of (oo)cysts purchased from Waterborne™ Inc. The IPR evaluation was performed by analyzing four samples of reverse osmosis water spiked with 1.2 x 10^4 *Cryptosporidium* oocysts and 4 x 10^4 cysts of *Giardia* in 10 L of the purified water. The MS was performed on the surface and reused water samples using the same inoculum size. Samples were processed in two 10-L aliquots: one contaminated with the standardized (oo)cysts suspension (experimental seeding) and the other without the respective contamination (blank). The inoculum values equivalent to 1.65 × 10^5 GC for oocysts and 1 × 10^8 GC for cysts obtained from the qPCR results were calculated and converted into the number of genomic copies per liter (GC/L) and used in the performance evaluation of the method. The recovery percentages of the (oo)cysts quantification method in both water types (purified and matrix) were calculated according to the equation below:

$$\%\ Recovery = \frac{(C1 - C2)}{C3} * 100 \quad (3)$$

Where:

C1 = (oo)cysts DNA copy number on the contaminated sample (experimental seeding)

C2 = (oo)cysts DNA copy number on the non-contaminated sample (blank)

C3 = (oo)cysts DNA copy number on the suspension added to the sample.

2.3.3.5. Nested PCR. Species characterization was performed by nested PCR and sequencing based on the *18S rRNA* genes for *Cryptosporidium* spp. and multilocus analysis of the *SSU rRNA*, *gdh* and *tpi* genes for *Giardia intestinalis* (Table S3 - Supplementary material). The nPCR for *Cryptosporidium* speciation was performed according to the conditions and primers described by Silva et al. (2013), which amplify a 611 pb fragment. The detection of *Giardia intestinalis* by the *SSU rRNA* gene was performed under identical reaction conditions, amplifying a 292 bp fragment as described by Appelbee et al. (2003).

For amplification of the glutamate dehydrogenase (*gdh*) gene fragment, a 530 bp product amplification was carried out as described by Cacciò et al. (2008). For the triosephosphate isomerase (*tpi*) gene, the same conditions described by Sulaiman et al. (2003) were used for both the first and second reactions.

The samples amplified in the second nPCR reactions for both protozoa were subjected to agarose gel electrophoresis. The fragment sizes were estimated by comparison with the 100bp Plus DNA Ladder (Invitrogen™). The gel was visualized in UV light and photodocumented (Epi Chemi II Darkroom, UVP). The amplified products of each positive sample were purified and sequenced by Sanger's method.

2.3.3.6. Cloning. For the separation of mixed species, a plasmid vector cloning step was added. After identification and purification of the sequence of interest, the insert was subjected to ligation in TOPO TA Cloning® Kit (Invitrogen™) vector and transformed into One Shot® TOP10 Electrocomp™ *E. coli* (Invitrogen™) electrocompetent cells, according to the manufacturer's instructions, except for a 10-min centrifugation step at 14,000 rpm of the ligation reaction prior to transformation. Analyses of transformants were performed after extraction of plasmids by a commercial kit, PureLink® Quick Plasmid Miniprep Kit (GE HealthCare, UK) and the M13 forward and reverse primers were used for the amplification and sequencing of the vector insertion region.

2.3.3.7. Sequencing and phylogenetic analysis. The sequencing reactions were commercially conducted by Genomic Engenharia Molecular (São Paulo, Brazil; https://genomic.com.br/). Quality control of the reactions was performed by the company, using pGEM 3Zf (+) and primer M13 (−21) provided by kits specific for sequencing reactions. The sequences were aligned at the NCBI BLAST program (Basic Local Alignment and Search Tool) for comparison with homologous sequences in the NCBI GenBank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The consensus sequences were aligned using Clustal W and BioEdit Sequence Alignment Editor tools (Hall, 1999). Phylogenetic analyses were performed using the Mega software program version X (Phoenix, AZ) (http://www.megasoftware.net/m_test_reliab.html) (Kumar et al., 2018) and evolutionary distances were calculated by the Maximum Likelihood method with Kimura 2-parameter and Tamura-Nei models, using Gamma distribution. A total of 1000 pseudo-replicas were used to support variability in statistical inferences. The generated sequences were deposited in Genbank under the numbers MW233035 to MW233039 for *Cryptosporidium 18S rRNA* gene, and the accession numbers for *Giardia* are: MW238477.1 for the *SSU rRNA* gene, MW344026 to MW344031 for *gdh* and MW344031 to MW344041 for *tpi*.

2.4. Statistical analysis

Data obtained were analyzed using R language and environment for statistical computing (R Core Team, 2020).

Concentrations of (oo)cysts below the limit of detection (LOD) were substituted with LOD divided by the square root of 2. In case of lack of results for the physical-chemical parameters in some sampling campaigns, these data were estimated using the multivariate technique of Principal Component Analysis – PCA using the EM (Expectation-Maximization) method (Josse and Husson, 2016).

Multivariate analyses were performed using normalized data, eliminating issues related to variable scales (units of measurement). Principal Component Analysis for Mixed Data – PCAMix was applied to evaluate the importance of the variables investigated in this study (matrix, location, physical, chemical and biological variables) in the protozoan data. For Cluster Analysis (Struyf et al., 1997), the Dissimilarity Matrix was used with the metric proposed by Gower (1971), which is based on the general dissimilarity coefficient, and applies to all types of random variables. The significance level adopted in all tests was 5%.

3. Results

3.1. Microbiological evaluation

The concentrations of microbial indicators are shown in Table 1. *E. coli* results (1.8x10^5 - 6.3 × 10^5 CFU/100 mL) at Cotia River (COTI3900) were one log higher than in Ribeirão dos Cristais (CRIS3400) samples (6.3x10^3 to 5.4 × 10^4 CFU/100 mL), and none met the São Paulo State *E. coli* standard for surface water supply source, 600 CFU/100 mL (Fig. S5 – Supplementary material). *Clostridium perfringens* data presented the same pattern of *E. coli*, with concentrations more elevated at COTI3900 (4.2x10^2 - 5.9 × 10^3 CFU/100 mL) in comparison

to CRIS3400 ($5.7x10^1$ – 8.2×10^2 CFU/100 mL) (Fig. S5 – Supplementary material).

In the Barueri (WWTP1), São Miguel (WWTP2), and Parque Novo Mundo (WWTP3) the absence of *E. coli* in most samples was expected, as the reuse water is chlorinated at the endpoint of the treatment process, easily eliminating these bacteria. *C. perfringens* was detected in 50% (7/14) of the samples in concentrations ranging from 1 to 112 CFU/100 mL. WWTP2 presented expressive values for *C. perfringens* in June, August, and September 2019.

3.2. Detection and quantification of Giardia cysts and Cryptosporidium oocysts by direct immunofluorescence (IMS-FA)

The parasites (oo)cysts concentrations detected at the two surface water sites and three reuse water plants, using IMS-FA methodology, are shown in Table 1 (Figs. S5 and S6 – Supplementary material). The percentages obtained in the initial precision and recovery (IPR) tests were 52.5% for *Giardia* cysts and 65.0% for *Cryptosporidium* oocysts, respectively, similar to the mean obtained in our routine assays since 2016 (*Giardia*: 54.7%; *Cryptosporidium:* 61.0%). The matrix spike (MS) provided recovery percentages ranging from 68% (COTI03900) to 32% (WWTP2) for cysts, and 62% (COTI03900) to 36% (WWTP3) for oocysts (Table S4 - Supplementary material). Although the quality controls of the quantification analysis of both protozoa met the criteria established by USEPA (2012), it is important to highlight that, in this study, the matrix recovery test (MS) was performed on only one sample from each collection site, and the recovery data for these sites in our laboratory routine are scarce. Therefore, these recovery rates should be considered with caution, especially if they are used to correct results expression, an approach not applied in this study.

Cryptosporidium spp. was present in 69.2% (9/13) and *Giardia* spp. in 100% (13/13) of surface water samples for public supply. *Cryptosporidium* oocysts were found at the lowest concentrations (0.1 and 41 oocysts/L) compared with *Giardia* cysts (7.2–354 cysts/L). The highest value of *Cryptosporidium* spp. was detected at site COTI03900 (sample 11A) with 41.2 oocysts/L in September 2019, while the highest density of *Giardia* spp. was observed at site CRIS03400 (sample 6A) with 354 cysts/L in May 2019. Samples 3A, 4A, 11A, and 13A, all belonging to the Cotia River (COTI03900), showed concentrations greater than 3.0 oocysts/L.

Regarding the reuse of water samples (Table 2 and Fig. S7), 85.7% (12/14) were positive for *Cryptosporidium* and *Giardia*, with concentrations ranging from 0.4 to 100.6 oocysts/L and 1.2 and 93.5 cysts/L, respectively. It is important to highlight the high counts of (oo)cysts found in Barueri WWTP1 in the February campaign, corroborated by the increase in turbidity (29.6 NTU). São Miguel WWTP2 also presented expressive values for both protozoa.

3.3. Detection and quantification of Giardia cysts and Cryptosporidium oocysts by qPCR

Recovery of protozoa in reverse osmosis water enriched with known concentrations of (oo)cysts was 25.5% (SD ± 9.4) for *Cryptosporidium* and 52.8% (SD ± 14.6) for *Giardia*. The effect of the matrix on the recovery of (oo)cysts using the qPCR technique was more negative for surface water samples (*Cryptosporidium,* 17.4%; *Giardia*, 7.6 %) when compared to reuse water (*Cryptosporidium*, 29.2%; *Giardi*a, 60.6%). Considering the acceptance criteria established by USEPA for initial precision and recovery (IPR) and the matrix spike recovery (MS) for immunofluorescence assay (USEPA, 2012) the percentages of recovery (IPR and MS) obtained in the qPCR assays for *Cryptosporidium* were below the values required. Absolute quantitation results by qPCR for surface and reuse water samples reported in this study were not corrected for recovery rates (Table S5 - Supplementary material).

Standard curve results for the *18S rRNA* gene of *C. parvum/C. hominis* showed the range of linear correlation values were E = 96.1%; R2 = 0.998; Slope = −3.41 and the limit of quantification (LOQ) for this gene was 0.25 CG/μL. For the *SSU rRNA* gene of *Giardia intestinalis*, the linear

Table 2
Concentration of *Cryptosporidium* oocysts and *Giardia* cysts per liter by qPCR assay in surface and reuse water samples.

Water source	Sample	Month/year	*Cryptosporidium parvum/hominis*			*Giardia intestinalis*		
			Cq values	GC/L^{-1}	oocysts/L*	*Cq* values	GC/L^{-1}	cysts/L*
CRIS03400	1A	Nov/2018	36	69.0	3.5	26	$5.0x10^4$	104.0
	2A	Feb/2019	36	67.9	3.4	31	$1.4x10^3$	2.9
	5A	Mar/2019	36	66.2	3.3	33	160.0	0.3
	6A	May/2019	38	2.5	0.1	23	$3.1x10^5$	639.0
	9A	July/2019	38	2.5	0.1	26	$2.8x10^4$	59.1
	10A	Sept/2019	38	2.5	0.1	33	756.0	1.6
	12A	Nov/2019	38	2.5	0.1	30	$3.8x10^3$	8.0
COTI03900	3A	Feb/2019	37	43.1	2.2	30	$5.6x10^3$	11.7
	4A	Mar/2019	36	65.1	3.3	26	$1.5x10^4$	30.9
	7A	May/2019	36	69.0	3.5	26	$3.5x10^4$	73.7
	8A	July/2019	34	55.9	2.8	23	$2.6x10^5$	549.0
	11A	Sept/2019	34	291.2	14.6	23	$1.3x10^5$	273.0
	13A	Nov/2019	34	194.9	9.7	23	$1.3x10^5$	266.0
WWTP1	1R	Nov/2018	36	77.4	3.9	30	$6.2x10^3$	12.8
	3R	Feb/2019	34	531.7	26.6	26	$4.4x10^4$	92.5
	5R	Apr/2019	38	3.8	0.2	31	$3.5x10^3$	7.4
WWTP2	2R	Nov/2018	36	77.8	3.9	38	152.0	0.3
	4R	Feb/2019	37	32.1	1.6	33	580.0	1.2
	6R	Apr/2019	37	14.6	0.7	31	$4.2x10^3$	8.7
	7R	June/2019	36	2.5	0.1	31	$4.0x10^3$	8.2
	10R	Aug/2019	37	48.6	2.4	32	$1.4x10^3$	3.0
	12R	Sept/2019	38	2.5	0.1	31	$6.3x10^3$	12.8
	14R	Dec/2019	36	77.4	3.9	26	$1.5x10^4$	30.3
WWTP3	8R	July/2019	36	69.7	3.5	27	$2.1x10^4$	43.9
	9R	Aug/2019	37	75.9	3.8	31	$1.6x10^3$	3.4
	11R	Sept/2019	38	2.5	0.1	33	513.0	1.1
	13R	Dec/2019	34	344.0	17.2	26	$1.2x10^4$	24.6

CRIS03400 = Ribeirão dos Cristais. COTI03900 = Baixo Cotia; WWTP: Wastewater Treatment Plant. WWTP1 =Barueri; WWTP2= São Miguel; WWTP3 = Pq. Novo Mundo; GC/L = Genome copies per liter. * GC/L were converted in (oo) cysts/L considering the number of genomic copies per (oo)cyst (20 copies to *Cryptosporidium* and 480 copies to *Giardia*).

correlation values were E = 97.3 %; R2 = 0.996; Slope = −3.39, and the LOQ was 16 GC/µL.

The results of quantification of *Cryptosporidium parvum/hominis 18S rRNA* and the *Giardia intestinalis SSU rRNA* genes in surface and reuse water samples are displayed in Table 2 and Fig. S7 (the equation used for the conversion of values is described in section 2.5.3). The oocyst numbers varied from 0.1 to 14.6/L in surface water and 0.1 to 26.6/L in reuse water. The highest values of these protozoa were detected in samples 11A and 13A from Cotia River, (COTI03900), and samples 3R from Barueri (WWTP1) and 13R from Pq. Novo Mundo (WWTP3).

The concentrations of *Giardia intestinalis* ranged from 0.3 to 639.8 cysts/L in surface water and from 0.3 to 92.5 cysts/L in reuse water. Catchment samples 6A (CRIS3400), 8A, 11A,13A (COTI03900), and 3R (WWTP1), showed the highest concentrations of *Giardia* cysts per liter, corroborating the immunofluorescence assay. This concordance was also observed for the lowest concentrations of cysts in both techniques (IMS-IFA and qPCR) in samples 5A (CRIS3400) and 2R (WWTP2). The values of *Cryptosporidium* oocyst concentrations were lower when compared to the results obtained in IMS-FA assays, however, it is possible to verify that there is a proportionality relationship with the densities of (oo)cysts found in the different samples, in both matrices.

3.4. Genotyping

3.4.1. Cryptosporidium species

The *18S rRNA* gene fragment (611bp) was amplified by nested PCR in 23% (3/13) of the surface water samples. Only sample 13A (COTI03900) resulted in high-quality sequencing results, which showed homology with *Cryptosporidium muris* (AF039498) when compared with available *Cryptosporidium* sequences in the GenBank database. In reuse water, the amplification was possible in 21.4% (3/14) of samples. Sample 7R (São Miguel - WWTP2) presented a mixed profile sequence (visualized through multiple peaks at the same position in the electropherogram) and was submitted to cloning to separate the species. Cloning analyses resulted in two sequences named 7R and 7Rb, which showed high homology with *Cryptosporidium muris* and *Cryptosporidium hominis*, respectively, the last one from Brazil (Ulloa-Stanojlović et al., 2016). Sample 12R (São Miguel -WWTP2) also showed homology with *C. muris* and the 13R (Pq. Novo Mundo WWTP3) sequence matched *C. parvum*. The results of the analyzed clusters are shown in Fig. 2, through the phylogenetic tree for *Cryptosporidium* spp.

3.4.2. Giardia intestinalis assemblages

Nested PCR amplification of at least one *Giardia intestinalis* gene was observed in 46.1% (6/13) of surface water samples and 50% (7/14) of reuse water samples.The *SSU rRNA* gene was detected in 23.1% (3/13)

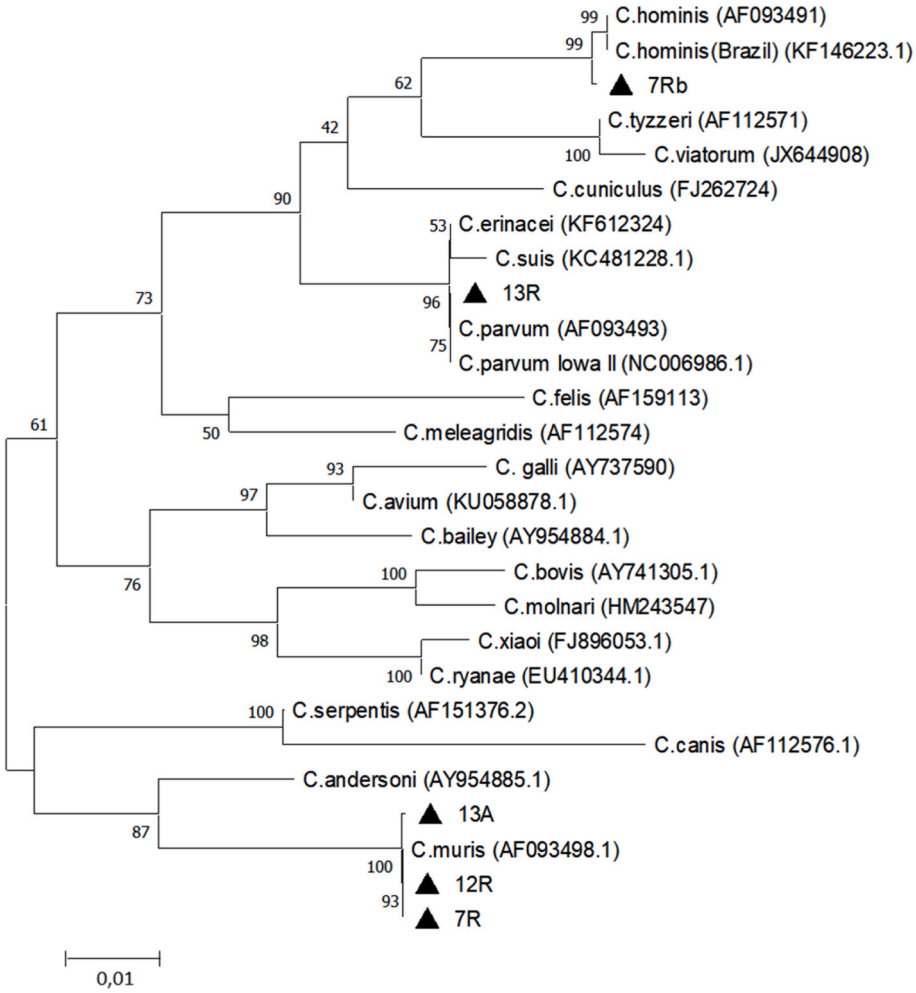

Fig. 2. Phylogenetic relationship obtained with *Cryptosporidium* spp. *18S rRNA* gene sequences inferred by the Neighbor Joining method using 1000 pseudo-replicates. The sequences obtained in this study are marked with a triangle, surface water: 13A and reuse: 7R, 7Rb, 12R, 13R. Reference sequences for each species are named by their respective accession numbers in NCBI database. Samples included in the phylogenetic tree were deposited in GenBank and can be accessed by numbers MW233035 to MW233039.

of the surface water samples, but it was only possible to sequence one sample, characterized as assemblage A (GenBank accession number: MW238477.1). There was no amplification of *SSU rRNA* gene in reuse water. For *gdh* gene, 23% (3/13) of surface water samples were positive for assemblage A (AII) and 28.5% (4/14) of reuse water samples showed homology with assemblage B (BIII and BIV). The *tpi* gene was the most frequent in this study, present in 46.2% (6/13) and 28.5% (4/14) of the surface and reuse water samples, respectively. Sequencing analysis identified assemblages A (AII) and B (BIII) in surface water and A (AII) and B (BIV) in reuse water. Additionally, only two samples yielded positive sequences for all three genes studied, underscoring the importance of a multilocus approach for identifying potential reservoirs in the environment.

In Figs. 3 and 4, it is possible to evaluate the phylogenetic reconstruction performed for the *gdh* and *tpi* genes, which made it possible to identify the assemblages and sub-assemblages, revealing the potential sources of contamination in the different matrices evaluated in the study.

3.5. Statistical analyses in watersheds and wastewater treatment plants

3.5.1. Physicochemical and bacteriological parameters

The data obtained for the biological and physicochemical variables in surface and reuse water samples can be found in Tables S6–S8 (Supplementary material).

Fig. 5 illustrates the significance of physicochemical and bacteriological variables in explaining the variability observed in the study. It demonstrates that the most important factor that explains the variability of the results is the matrix, that is, the results of the concentrations of protozoa in the surface water supply sources differ significantly from those of the reuse water of the WWTPs. High numbers of *Giardia* spp. cysts are not expected in reuse water, since they undergo a treatment process, and therefore their presence indicates that the treatment is not effective in removing these protozoa.

The second most important variable that differentiates the matrix is the presence of *E. coli*, while the location is the third most important factor, that is, even in the same matrix, there may be differentiated behavior by location. *Cryptosporidium* and *Clostridium* are variables that make this differentiation possible. The presence of *Giardia* cysts better discriminated the reservoirs, being, therefore, an important indicator of water contamination.

3.5.2. Analysis by samples in surface and reuse water

Fig. 6 presents the cluster analysis, showing three distinct groups marked by different colors, along with one isolated sample (3R) from Barueri WWTP1, which exhibits significantly higher (oo)cyst concentrations compared to the other samples. This scenario clearly shows operational problems at the reuse plant, which led to its interruption in operation for maintenance. The first cluster (cyan) is formed by samples 13A, 11A, 3A and 7A, from Baixo Cotia River (COTI03900), which showed high amounts of *Giardia*, *Clostridium*, *E. coli* and *Cryptosporidium*. Gray cluster concentrated reuse water samples and the green cluster, most samples from Ribeirão dos Cristais river (CRIS03400). The degree of contamination decreases from left to right.

4. Discussion

This study involved assays for detection, quantification, and speciation of *Giardia* and *Cryptosporidium* in two rivers used as source water for Water Treatment Plants (DWTPs), and three Wastewater Treatment Plants (WWTPs) producing treated reuse water in the city of São Paulo. Data on the occurrence and identification of these protozoa are crucial for determining contamination sources, evaluating removal efficiency, and implementing water safety and disease prevention measures. The universally recommended methodology for the detection of protozoa in water matrices uses filters and reagents that help to reduce the impact of turbidity in the recovery of (oo)cysts, as well as monoclonal antibodies for purification and isolation, thus requiring a rigid process of quality control.

The recovery and detection of (oo)cysts in environmental matrices represents a challenge for most water and sewage analysis laboratories, due to the great complexity of these samples. Determining the recovery of standard concentrations of (oo)cysts in pure water and the evaluated matrices is essential to guarantee the performance of the analysis

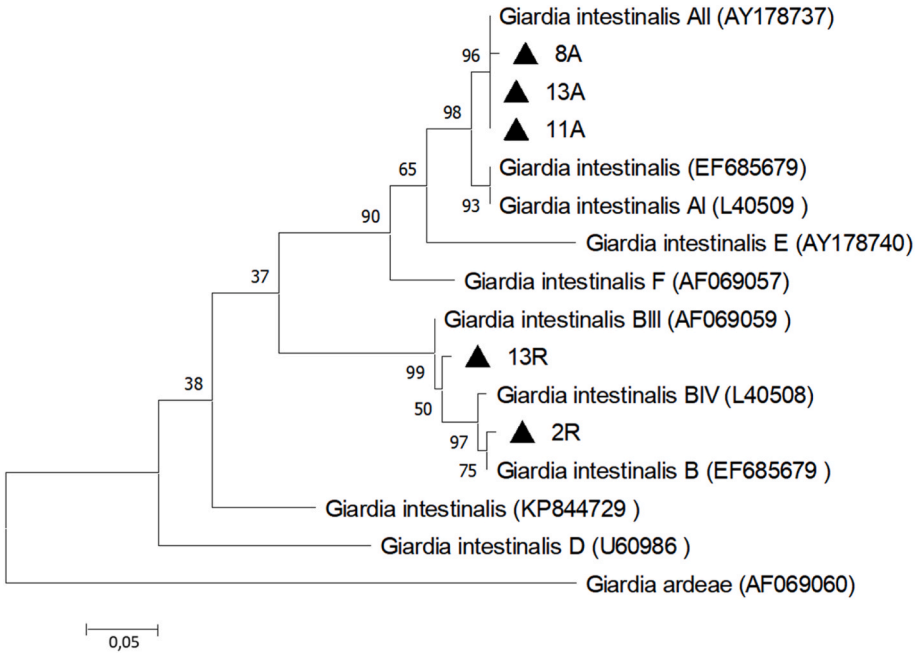

Fig. 3. Phylogenetic relationship among *Giardia intestinalis* assemblages using *gdh* sequences, inferred by the Neighbor Joining method using 1000 pseudo-replicates. The sequences obtained in this study are marked with a triangle, surface water: 8A,11A, 13A and reuse: 2R,13R. Reference sequences for each species are named by their respective accession numbers in NCBI database. Samples included in the phylogenetic tree were deposited in GenBank and can be accessed by numbers MW344026 to MW344030.

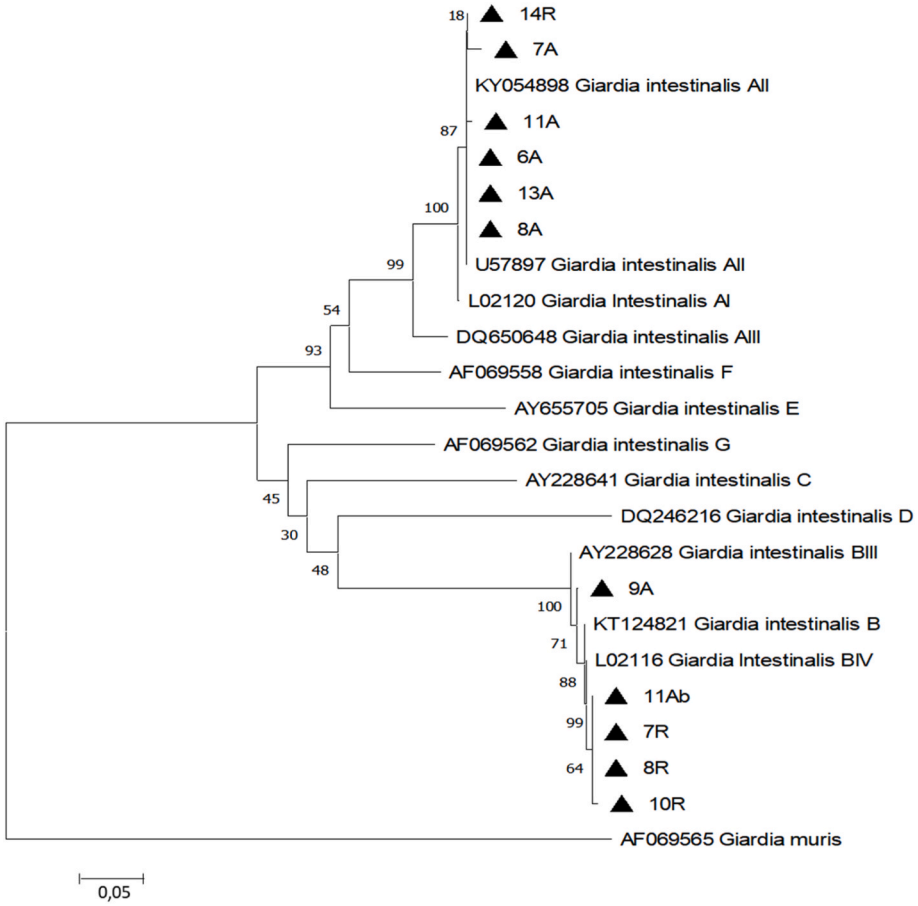

Fig. 4. Phylogenetic relationship among *Giardia intestinalis* assemblages using *tpi* sequences, inferred by the Neighbor Joining method using 1000 pseudo-replicates. The sequences obtained in this study are marked with a triangle, surface water: 6A,7A, 8A, 9A,11A,11AB,13A and reuse: 7R, 8R,10R,14R. Reference sequences for each species are named by their respective accession numbers in NCBI database. Samples included in the phylogenetic tree were deposited in GenBank and can be accessed by numbers MW344031 to MW344041.

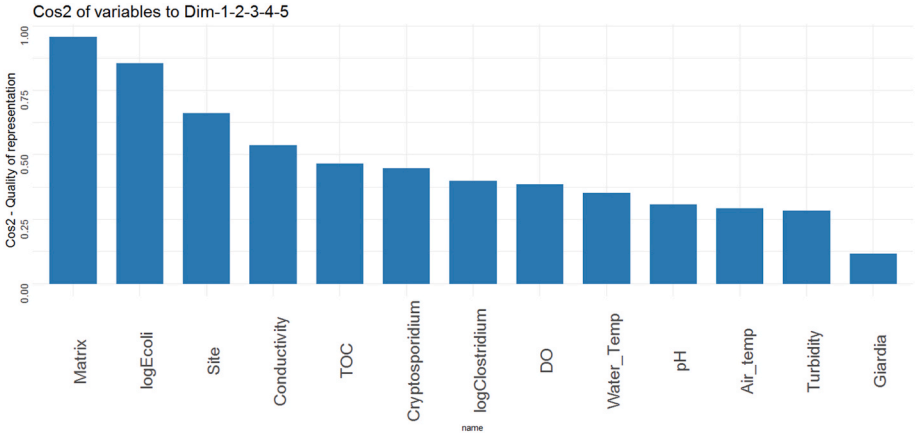

Fig. 5. Graphic representation of physicochemical and microbiological variability (High cos2 values indicate a good representation of the variable in the five principal components; low cos2 values indicate that the variable is not well represented).

(USEPA, 2012). In the present study, the initial precision and recovery (IPR) and the spiked matrix recovery (MS) performed for IMS-FA tests met the quality control acceptance criteria recommended by the United States Environmental Protection Agency (USEPA) for Methods 1623.1 and 1693 (Table S4 - Supplementary material).

The conversion of GC/L to (oo)cysts/L made it possible to apply the same USEPA quality control criteria of the IMF-FA method to qPCR, but the results showed lower performance in both initial (IPR) and matrix (MS) recovery. The acceptance criterion was met only for *Giardia* recovery in reverse osmosis water and in the WWTP2 matrix. Despite the limitations of these comparisons, since this evaluation for method performance was established for IMS-FA, not qPCR, this difference, especially for MS, can be attributed to the low number of (oo)cysts recovered or the presence of inhibitors in the samples, which is critical for PCR performance, suggesting interference in DNA amplification.

Different factors may be associated with the loss of (oo)cysts when

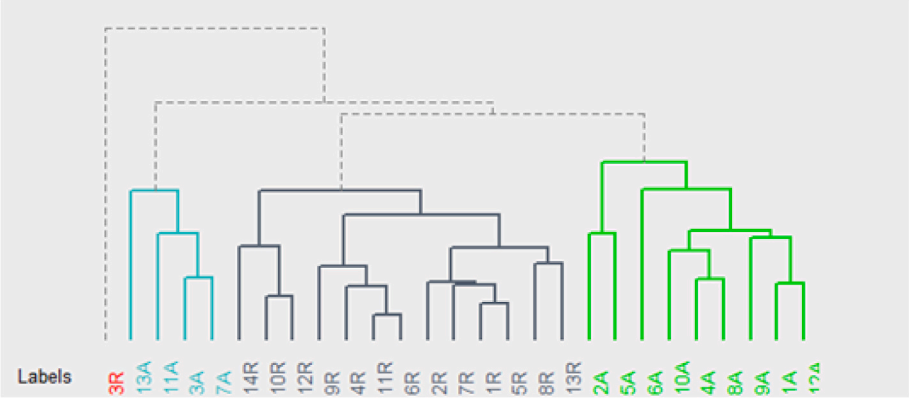

Fig. 6. Dendrogram showing the clusters from surface and reuse water samples.

processed in multiple steps. Some characteristics of aquatic matrices, such as greater adsorption of suspended particles with different properties found in tributaries and effluents of water and sewage treatment systems, may also be associated with lower recovery efficiency in assays (Yang et al., 2013; Masangkay et al., 2020). According to Ongerth and Saaed (2013), negative analytical results obtained from processing samples with reduced volumes, limited to 10 L, and based on the recovery efficiency method, can be considered biased. This is because oocysts are present in water sources intermittently and may occur in low concentrations at the time of sampling. The authors recommend increasing the volume of processed samples; this approach, despite the higher cost of analysis, can significantly reduce the frequency of results below the detection limit.

Quantitative PCR performance evaluation data on environmental samples is important but limited. Some studies suggest that the percentage of recovery may vary according to the type of sample, concentration methodology, and the parameters analyzed (Masangkay et al., 2020). In addition, inhibitory substances (humic acids, fats, polyphenols, heavy metals), extraction of nucleic acids, and the composition of the cell wall of (oo)cysts, which makes access to genetic material difficult, can reduce the number of isolated organisms and impair the detection success (Jiang et al., 2005; Plutzer et al., 2008; Yu et al., 2009; Yang et al., 2013).

This study also evaluated the inhibitory effects that could prevent DNA amplification in qPCR and generate false-negative results. However, no inhibitory changes (Cq $\geq$ 0.5) were detected in the IPC (Internal Positive Control) reactions (unpublished data). Fontaine and Guillot (2003), using the Envirochek® capsule filtration method and qPCR, observed the detection limit of five oocysts spiked in 20L and 100L of tap water, and eight oocysts in 5L of surface water. The IPR assays provided a percentage of 47%, while in matrices the recovery of oocysts was 12%, corroborating the percentages observed in our study.

There is no recommended or universally used molecular method for the detection and quantification of protozoa in environmental samples. Galvani et al. (2019) evaluated the presence of *Toxoplasma gondii* oocysts in the same watershed surface waters evaluated in this study, also using qPCR and the recovery tests with experimental seeding, which reached the maximum result of 8.9% for MS.

Our results showed that both surface water sources for public supply and treated wastewater supplied for urban reuse are contaminated with (oo)cysts, demonstrating the potential of applying qPCR assays to evaluate contaminated water.

It is important to note that IMS-FA is the gold standard technique for environmental monitoring of *Cryptosporidium* spp. and *Giardia* spp. These assays are based on morphological analysis and the results are reported at the genus level, while the qPCR analysis detects species using specific probes. Therefore, in IMS-FA other non-*C. parvum/hominis* species can be detected, as demonstrated in the species molecular characterization assays, where cloning and sequencing methods were applied to a sample with mixed species. On the other hand, although the presence of multicopy genes, such as *18S rRNA* and *SSU rRNA*, can increase the sensitivity of target detection, it is important to note that crucial steps such as DNA extraction and (oo)cyst recovery methods can impact the detection of genomic copies (GC) by qPCR (Mthethwa et al., 2022; Hachimi et al., 2024).

The presence of both protozoa in surface and reused water samples in the present study can be attributed to the low rates of sewage collection and treatment in the watershed region, as well as the low efficiency of the treatment used in the wastewater treatment plant.

The microbiological data from Cotia river and Ribeirão dos Cristais river reported in our study (Table 1, Fig. S5) showed a worrying scenario for the health and environmental surveillance systems concerning fecal indicator bacteria, *E. coli* and *Clostridium perfringens*, confirming the release of inadequately treated domestic effluents into water sources and the need to continuously monitoring our watersheds (BRASIL, 2021). This data is reenforced by the protozoa results (Fig. S5). On the other hand, the absence of *E. coli* was expected in most reuse water samples, as it is chlorinated at the end of the treatment process, easily eliminating these bacteria, while *C. perfringens* was detected in 50% (7/14) of the samples at concentrations ranging from 1 to 112 CFU/100 mL, showing that this bacterium can be a better indicator of the presence of protozoa in this matrix. The high percentage of positive for *Giardia* and *Cryptosporidium* in these samples shows the weakness of these fecal indicator bacteria. of protozoa.

Samples from the Cotia River, collected at the Drinking Water Treatment Plant intake, showed significant quantification of *Cryptosporidium* and *Giardia*, both by qPCR and by IMS-FA assays (Tables 1 and 2, Figs. S5 and S6). The microbiological results and statistical data for cluster analysis confirmed this reservoir's contamination and poor water quality, with great concern for the protozoan *Giardia*. This water resource has been monitored and evaluated annually for the presence of pathogenic microorganisms, and in recent decades it has been degraded due to anthropic action, thus interfering with the water quality The implementation of advanced treatment processes has already been proposed by environmental agencies to ensure the removal of these pathogens in drinking water, however, to date there is no legal requirement or regulation for this.

Samples 3A, 4A, 11A, and 13A, all belonging to the Cotia River (COTI03900), showed concentrations greater than 3.0 oocysts/L and high values for all microbiological indicators evaluated, indicating a potential risk of infection for the population. However, the Cotia River (COTI03900) was deactivated in 2019 and, according to the Annual Report on the Quality of Inland Waters at the State of São Paulo, this point that belongs to Cotia River is no longer used for public supply purposes (CETESB, 2020).

The presence of oocysts and cysts in surface water supply, DWTP,

and WWTP has been highlighted in many studies from different Brazilian cities (Sato et al., 2013; Franco et al., 2016; Coelho et al., 2017; Araújo et al., 2018; Leal et al., 2018; Fantinatti et al., 2020; Breternitz et al., 2020; Zini et al., 2021; Scherer et al., 2022; Keller et al., 2024). Data obtained in the present study is parallel with a recent report by Keller et al. (2024), on surface water destined for public supply in the state of Goiás, Brazil, where they identified a high frequency of *Giardia* cysts (75–100%) and *Cryptosporidium* oocysts (67–83%).

Nonetheless, a lower prevalence of these protozoa was observed in a study conducted in the surface water of eleven municipalities in the state of São Paulo where a prevalence of 29.7% of cysts and 30.4% of oocysts was reported in the 128 samples evaluated (Breternitz et al., 2020). These significant studies support our findings and emphasize the need to implement effective barriers and protection measures for watersheds in large urban centers.

The high frequency and concentration of *Giardia* cysts and *Cryptosporidium* oocysts observed in the reused water from the three monitored WWTPs (Tables 1 and 2, Figs. S5 and 7), and the absence or the relatively low values of fecal microbial indicators demonstrate the fragility of these indicators in assessing the quality of reuse waters and emphasize the importance of mechanical removal in the treatment process. The microbiological quality of urban reuse water has been also monitored in two WWTPs by Razzolini et al. (2020) in the city of São Paulo and, the *Cryptosporidium* and *Giardia* concentrations, measured by the USEPA Method 1623.1, were compatible with those found in our study (<0.03–16 cysts/L and <0.03 oocyst/L – 25.8 oocysts/L).

In general, genetic amplification and characterization assays are useful both for environmental monitoring and differentiation of species of interest. The detection results of (oo)cysts and speciation observed in previous studies corroborate our findings and reveal that the occurrence of species that circulate in the Brazilian environment may be underestimated (Coelho et al., 2017; Araújo et al., 2018; Durigan et al., 2018; Fernandes et al., 2011; Scherer et al., 2022; Zini et al., 2021).

Due to the sensitivity and specificity of detection by hydrolysis probes, qPCR assays have become indispensable tools in microbiological assessment for environmental matrices. As in different studies that used qPCR to detect *C. parvum* and *C. hominis* species, our study reaffirms the importance of establishing robust assays to specifically detect species and their subtypes that provide more accurate assessments of exposure risk (Yang et al., 2013; Kumar et al., 2016; Masangkay et al., 2020; Mthethwa et al., 2022; Hachimi et al., 2024).

The species and genotypes characterized in the present study are essential to support studies of water surveillance and safety water management (Figs. 2–4). The species *C. hominis* and *C. parvum*, and *Giardia intestinalis* AII, BIII, and BVI, were identified in both surface and reuse water samples. These species have been associated with waterborne outbreaks in different continents and represent a public health risk, in addition to triggering environmental and socioeconomic damages in the region. Thus, the desirable target is the absence of these pathogens in both waters intended for consumption and reuse. Yamashiro et al. (2019) detected *Giardia* assemblages C and BIV in raw sewage, and *Giardia* AII in a treated effluent sample from Campinas, a city located near the metropolitan region of São Paulo, and indicated that, in that region, contamination can come from both human and animal origins.

Evaluation by real-time PCR based on *18S rRNA* and *SSU rRNA* genes for speciation of *C. parvum* and *Giardia lamblia*, respectively, was performed on water samples from Thailand, Malaysia, and the Philippines. The authors concluded that the presence of these pathogens in treated or untreated raw water may result in a potential risk of zoonotic disease transmission to the population of the evaluated regions (Kumar et al., 2016).

In the present study, PCR assays combined with cloning and sequencing allowed the identification of important species circulating in surface and reuse waters. Amplification of DNA in the reuse water samples revealed the mixed pattern often found in environmental samples. After in silico analysis, the positive fragments showed a profile compatible with *C. hominis* and *C. muris*. Silva et al. (2017)have revealed that synanthropic rodents are naturally infected by *C. muris* and are responsible for transmitting numerous diseases to humans (Fig. 2). Furthermore, wild rodents also become a potential host option for some genotypes, revealing a complex chain of transmission between wild animals (Xiao et al., 2000). The species detected in the reuse water samples imply the need for monitoring and evaluation for the effective removal of these pathogens in waters intended for different reuse purposes, potable and non-potable, which can represent a risk for the occurrence of enteric diseases.

According to the SNIS (National Basic Sanitation System), only 52.2% of the sewage generated in the country is treated and the risk of infections associated with sewage discharged into the environment is high. Even before the COVID-19 pandemic began in Brazil, poor sanitation was already overloading the health system with 273.403 hospitalizations for waterborne diseases and an incidence of 13.01 cases per 10.000 inhabitants (BRASIL, 2023).

In Brazil, as in other Latin American countries, reports of outbreaks by *Cryptosporidium* and *Giardia* through water and food intake are limited and certainly underestimated. Many people still live in places without access to basic sanitation and surface waters continue to receive sewage without treatment or poorly treated. The results of this study, corroborated by data from other studies in the region, demonstrate a high circulation of these protozoa in the environment and consequently a risk to human health.

Water quality standards for human consumption in the country are established in the National Ordinance GM//MS N° 888/2021 (BRASIL, 2021). The current water potability standard emphasizes the monitoring of *Cryptosporidium* and *Giardia* protozoa in the water supply, making it mandatory at water collection points by treatment plants, following the principles of the Water Safety Plan (PSA), and requires research of (oo) cysts when the annual geometric mean of *Escherichia coli*/100 mL is greater than or equal to 1.000, to ensure the quality of drinking water and protect the health of the population. Therefore, our study could offer further insights into assessing the risk of exposure within the local population.

A challenge to be faced by health, sanitation, and environmental authorities given the high concentrations of these protozoa present in the surface water sources and Water Treatment Plants is the treatment and disposal of backwash water since (oo)cysts are concentrated in the filters during the filtration process. Specific regulations must be adopted to prevent this water from returning to the treatment system or being discharged into water bodies without adequate treatment.

It is also important to highlight that in Brazil, despite there being no national regulations for reuse water quality standards, the State of São Paulo implemented a Resolution that imposes restrictions for the direct non-potable reuse of water for urban purposes, from Sanitary Sewage Treatment Stations, with maximum limits established for cysts of *Giardia* and oocysts of *Cryptosporidium* for unrestricted reuse. (SES/SMA/SSRH, 2020).

Despite the significant contributions of this study to the understanding of the epidemiological surveillance of the protozoa *Cryptosporidium* and *Giardia* in surface and reused water, some limitations were identified. Among them, the limited number of spike matrices analyzed (MS) and the approach to obtain more homogeneous samples in the comparison of the different quantification methods, It would have been more appropriate to combine the samples after IMS and then separate the aliquots for the different assays. Such factors may have hindered a more comprehensive exploration of the complexities of the evaluated results. Future research could benefit from employing these methodologies to investigate these aspects in greater depth.

This study performed an integrated assessment of microbiological results at the sampled sites and revealed protozoan species with a high incidence in previously reported outbreaks, which may originate from both anthroponotic and zoonotic sources and indicate a risk of

contamination for the local population if they are not removed during treatment (Sato et al., 2013). This study reaffirms the importance of monitoring and seeking to better understand the presence and behavior of these intestinal parasites in the environment.

5. Conclusion

The results obtained herein showed that both surface water sources for public supply and treated wastewater supplied for urban reuse are contaminated with high concentrations of (oo)cysts of *Cryptosporidium* and *Giardia*, representing a high risk to the population health, and should be a primary concern for Health, Sanitation and Environmental authorities. The presence of *C. hominis* and *C. parvum*, as well as *Giardia intestinalis* assemblages AII, BIII, and BVI, in water used for public supply and treated wastewater provided for reuse from Sao Paulo city, indicate that contact with these matrices can offer a potential risk to the public health. Further infectivity studies should be encouraged to provide a complementary assessment of the risk of exposure to these species by the local population.

qPCR assays applied in the evaluation of contaminated water were able to detect and quantify both protozoa, highlighting that molecular assays are important tools for environmental surveillance. Despite the growing need for water reuse for non-potable purposes, the use in urban activities must be carried out carefully, due to the presence of pathogenic organisms that are difficult to remove and inactivate by conventional treatment methods. Finally, efforts to avoid contamination of water sources are essential and strategic.

Funding

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo – FAPESP, Brazil [grant number: 2017/10175-3].

CRediT authorship contribution statement

Ronalda Silva de Araújo: Writing – original draft, Project administration, Conceptualization. **Mikaela Renata Funada Barbosa:** Resources, Project administration. **Milena Dropa:** Writing – review & editing, Investigation. **Vanessa Cristina Araujo de Castro:** Investigation. **Ana Tereza Galvani:** Investigation. **José Antônio Padula:** Investigation. **Antônio de Castro Bruni:** Software, Formal analysis. **Carlos Jesus Brandão:** Resources. **Maria Anete Lallo:** Writing – review & editing, Conceptualization. **Maria Inês Zanoli Sato:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

The authors wish to acknowledge the staff of the Environmental Company of the São Paulo State (CETESB), especially for conducting the sampling and field analysis.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2024.125089.

Data availability

Data will be made available on request.

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