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High occurrence of viable forms of *Cryptosporidium* and *Giardia* in domestic sewage from an agricultural region of Brazil

Winni Alves Ladeia [a,*], Felippe Danyel Cardoso Martins [a], Beatriz de Souza Lima Nino [a], Arielle da Cunha Silvério [a], Ana Clécia da Silva [a], Raul Ossada[b], Douglas Aparecido da Silva [a], João Luis Garcia [a] and Roberta Lemos Freire [a]

[a] Preventive Veterinary Medicine Department, State University of Londrina, Celso Garcia Cid, PR 445 Highway km 388, University Campus, Londrina, Paraná 86057-970, Brazil
[b] Preventive Veterinary Medicine and Animal Health Department, Veterinary Medicine and Zootechnics College, São Paulo University, Professor Orlando M de Paiva Avenue, 87, São Paulo 05508-270, Brazil
*Corresponding author. E-mail: winnialvesladeia@gmail.com

WAL, 0000-0002-8815-5773; FDCM, 0000-0002-9162-1536; Bde SLN, 0000-0001-7206-4466; A da CS, 0000-0003-3604-3021; ACd, 0000-0003-0295-6869; DAd, 0000-0002-6327-5242; JLG, 0000-0003-1826-7582; RLF, 0000-0001-8522-035X

ABSTRACT

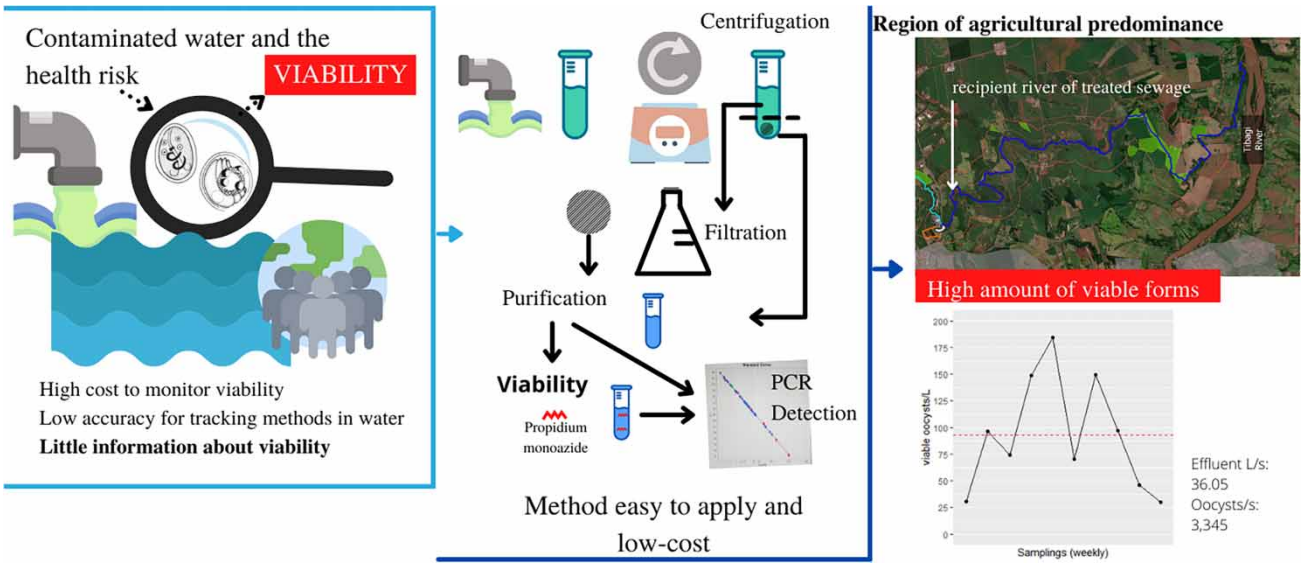
Cryptosporidium and *Giardia* are the main etiologies of waterborne outbreaks caused by protozoa. These parasites are commonly detected in wastewater; however, there is little knowledge about the concentration of viable forms in treated sewage, mainly in small communities. To understand more about the presence of viable oocysts and cysts in domestic sewage, we monitored the affluent and effluent of a wastewater treatment plant (WWTP) in inner-city Brazil. Ten samplings and seven follow-ups were performed in 2020. Samples were concentrated by centrifugation, filtration and purified by fluctuation. Viability was accessed by propidium-monoazide (PMA) associated with nPCR and qPCR. Both viable protozoa were detected in all raw sewage samples (average: 438.5 viable oocysts/L). Regarding treated sewage, *Cryptosporidium* was detected in all of the samples (average: 92.8 viable oocysts/L) and *Giardia* was detected in 70% with viable cysts in 30%. Considering the follow-ups, 31.17% of *Cryptosporidium* viable oocysts remained in the effluent after the treatment. High amounts of *Cryptosporidium* and a high frequency of *Giardia* were detected, therefore both arrived at WWTP and were discharged into the river. These alert the presence of agro-industrial effluents into domestic sewage and demonstrated the effectiveness of the concentration technique for monitoring protozoa in wastewater.

Key words: contamination, propidium-monoazide, qPCR, quantification, wastewater, WWTP

HIGHLIGHTS

- Viable oocysts and cysts persist after the stabilization pond treatment system.
- High concentration of viable *Cryptosporidium* was quantified in treated sewage.
- An accurate methodology to concentrate and purify (oo)cysts in wastewater was described.
- PMA-PCR is a cheap and efficient methodology to detect (oo)cysts viability.
- Agro-industrial effluents might influence protozoa concentration in domestic sewage.

GRAPHICAL ABSTRACT



1. INTRODUCTION

Cryptosporidium and *Giardia* are the most frequent protozoa etiologies of waterborne outbreaks worldwide. Both parasites are well known for a gastroenteritis etiology in their hosts, which leads to a moderate-to-asymptomatic condition in immunocompetent and a severe condition in immunocompromised individuals (Baldursson & Karanis 2011). These genera are widely distributed in the world mainly because of their low infectious dose (10–100 oocysts and cysts), the potential for indirect water and food transmission, the high parasitic loads eliminated in the feces (10^7 oocysts and 10^6 cysts), the broad variety of hosts, zoonotic potential and high resistance to both environmental conditions and conventional water treatments (Thompson & Ash 2016).

Characteristics of persistence and dissemination of *Cryptosporidium* associated with transmission by water led to its classification as a reference pathogen for water quality (Medema *et al.* 2006). These protozoa were found in all types of water; however, they present a higher frequency and concentration in wastewater, especially in domestic sewage (Nguyen *et al.* 2016). Sewage is a pool for excrement from the whole urban population, consequently, it is where all substances, metabolites and pathogens wasted by urine and feces converge. Sewage monitoring aims to better characterize the population's health and it has recently been used as an epidemiological tool (Xiao *et al.* 2018; Mao *et al.* 2020).

Wastewater-based epidemiology (WBE) shows promising results regarding the surveillance and prediction of diseases, and illicit habits that can harm the population's health. To predict these health threats in the population, analyses that detect and quantify pathogenic agents and chemical substances have been done in raw sewage (Mao *et al.* 2020). However, studies in treated sewage aiming to understand whether byproducts and pathogens can be released into the environment and their effect on ecosystems are scarce, especially regarding *Cryptosporidium* and *Giardia* because few effective diagnostic methodologies for viability have been established (Xiao *et al.* 2018).

To assess the magnitude of these parasites' release into the environment and possible damage to health, the viability of infectious forms in treated sewage needs to be verified (Ma *et al.* 2016; Martins *et al.* 2019). Methodologies for detecting the viability of these parasites have been described, although the accuracy is compromised when applied in environmental samples. Regarding this, molecular analyses present better sensibility and specificity than microscopic methodologies, but they demand sample purifying which increases costs. As an alternative for cost and performance, to verify viability in environmental samples, PMA-PCR is recently studied for diagnosis (Liang & Keeley 2012; Rousseau *et al.* 2018). To understand the environmental and health risks which may be caused by the environmental contamination with the viable (oo)cysts of *Cryptosporidium* and *Giardia*, this study aimed to verify the presence of total and viable oocysts and cysts of these protozoa in raw and treated sewage in a wastewater treatment plant (WWTP) in inner-city Brazil, and also to quantify total and viable *Cryptosporidium* oocysts that can reach the receiving water body.

2. MATERIALS AND METHODS

2.1. Study site description: socioeconomic and sanitation aspects

The study was conducted in Ibiporã, a municipality in the metropolitan region of Londrina located in the North Central mesoregion of Paraná State, Brazil (SIRGAS 2000: −23.2695; −51.0437) (Figure 1). In total, 54,558 inhabitants live in the municipality, which is ranked 94th position in the State regarding Human Development Index (HDI) (0.726) and has a hospitalizations-per-diarrhea rate of 1.7 per thousand inhabitants (Brazil 2019). The local climate is humid mesothermal, within the Atlantic Forest biome, with an average temperature ranging from 16.8 to 26.8 °C.

Sewage collection covers 96% of the municipality as well as the tap drinking water supply, which is obtained from surface water and treated by a secondary system. The collected sewage is treated in three distinct WWTP with a preliminary treatment consisting of railings, and a treatment system consisting of stabilization ponds that reach a hydraulic detention time of 14 days. The WWTP where the study was carried out is responsible for 56% of the city's collection and has three anaerobic lagoons and an optional lagoon oxygenated by algae. The average annual flow in 2019 was 171.57 m³/h for the affluent and 129.78 m³/h for the effluent released in the Jacutinga River (Brazil 2019).

2.2. Sampling

One sampling per week of the affluent and effluent of WWTP was carried out for 10 weeks. All samplings took place on Wednesdays from 2 September to 4 November 2020, except on 28 October, in which the sample was collected on Thursday, a fact that did not allow the monitoring of the treatment process between the seventh and ninth sampling. Thus, it was possible to follow up on the seven sewage treatments on arrival and discharge of wastewater of WWTP with respect to the 14-day

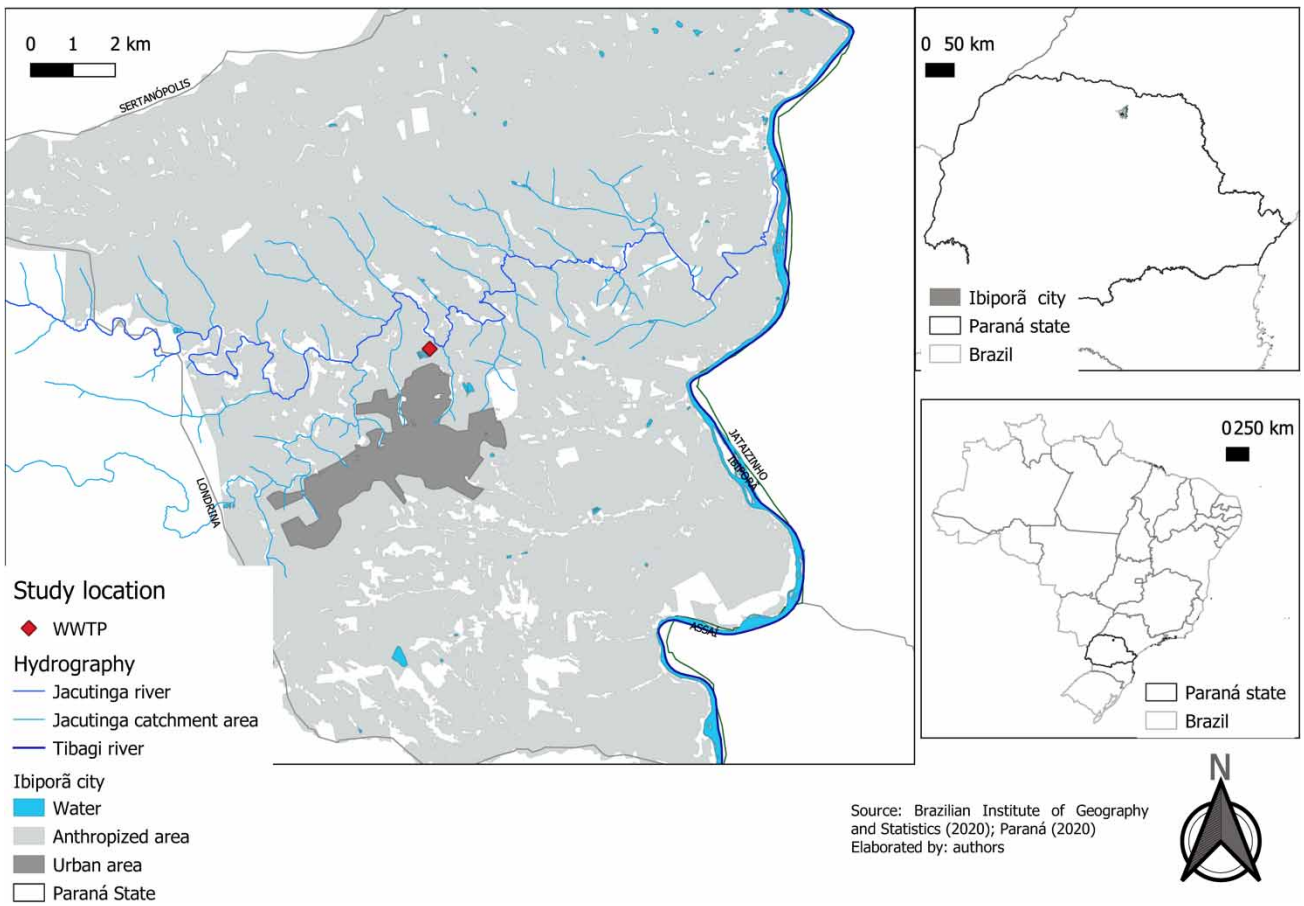


Figure 1 | Spatial description of affluent and effluent sampling sites (WWTP) from Ibiporã and the Jacutinga River Catchment, 2020.
Note: WWTP – wastewater treatment plant.

hydraulic detention time. Plastic gallons were used for sample packaging and transportation. Samples were obtained from the affluent after railing and from the effluent before the discharge into the water body. Gallons were packed in isothermal boxes with a temperature between 4 and 7 °C until the samples were processed. Turbidity measurement (NTU) was performed by an AP2000 turbidimeter (PoliControl, São Paulo, Brazil).

To disinfect the gallons, sodium hypochlorite (62.5 ppm) was added and left to act for 12 h. The solution was discarded, and 2% of the total gallon volume of 1% Tween 80 (100 mL) was added and the gallon was subjected to vigorous mechanical stirring for 5 min. The residual solution was discarded, and the container was rinsed to remove the foam. Then, they were subjected to three rinses with distilled water to remove the chemical residues (Martins *et al.* 2019).

2.3. Samples concentration and purification

Samples' concentration was performed by an association of centrifugation and filtration aiming to improve the recovery. Six hundred milliliters of the affluent and 400 mL of the effluent were centrifuged in two cycles at 1,500×*g* for 15 min, and the pellet was reserved until purification (Santos *et al.* 2011). From the supernatant of the first centrifugation, 200–400 mL of the affluent and 400 mL of the effluent were filtered through a cellulose ester membrane (1.2 μm porosity) coupled to a vacuum system. The residue retained on the membrane was scraped for 20 min into a shallow layer of 0.1% Tween 80 solution using plastic handles. The scraping product was centrifuged at 1,500×*g* for 15 min and the pellet was reserved until purification (Cantusio Neto *et al.* 2010).

The purification of the concentrate occurred by centrifugation–flotation in sucrose solution. Briefly, a conical tube (15 mL) was 85% filled with sucrose solution (density of 1.208 g/mL) and 15% with the pellets of concentrated samples. Tubes were subjected to centrifugation at 1,250×*g* for 10 min in a swing-type rotor (Kimura *et al.* 2000). The supernatant obtained was transferred to a 50 mL tube and distilled water was added to the total volume and tubes were centrifuged in two cycles at 1,500×*g* for 15 min to remove sucrose.

The resulting pellet was divided into two aliquots for raw and treated sewage to enable the verification of total and viable (oo)cysts. Each raw and treated sewage sample was divided into two aliquots – one aliquot without propidium-monoazide (PMA) (RS, Raw Sewage and TS, Treated Sewage) and another aliquot with PMA (RS-PMA, Raw Sewage with PMA and TS-PMA, Treated Sewage with PMA). These aliquots were stored at 4 °C for less than 24 h until staining with PMA.

2.4. PMA staining for viability assessment

PMA dye (Biotium, Inc., Hayward, CA, USA) was suspended in a 20% dimethylsulphoxide (DMSO) solution (Sigma Aldrich Co., St. Louis, MO, USA) to obtain a 2 mM stock solution concentration. For sample staining, 195 μL of the aliquots intended for the addition of PMA (RS-PMA and TS-PMA) were transferred to 1.5 mL microtubes and 15 μL of PMA (2 mM) were added, resulting in a concentration of 143 μM PMA per sample (Liang & Keeley 2012; Alonso *et al.* 2014).

For dye penetration into cells with a damaged membrane, the solution was kept in a light shelter at room temperature for 30 min with slow manual stirring every 5 min. Dye photoactivation was performed in microtubes deposited on ice and subjected to 100-W LED light (λ: 450–500), for 10 min, with slow mechanical agitation every 2 min for 30 s (Alonso *et al.* 2014). The solutions were centrifuged at 5,000×*g* for 10 min, the pellet was resuspended in distilled water and the samples were stored at −20 °C until DNA extraction (Liang & Keeley 2012).

2.5. Standard curve for *Cryptosporidium* qPCR

Oocysts from stool samples were quantified using a Neubauer chamber and Direct Immunofluorescence Assay (DIFA) by the Merifluor® *Cryptosporidium Giardia* kit (Meridian Bioscience, Cincinnati, OH, USA). Subsequently, *Cryptosporidium* oocysts (5.10^3 oocysts/μL) were pretreated with 70% ethyl alcohol for 5 min, to destroy sensitive microorganisms and concentrate at 5,000×*g* for 15 min. The pellet was resuspended in Tris-EDTA-HCl (pH 8.0) buffer and the DNA extraction was performed by DNeasy® PowerSoil® kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol with the addition of a freezing (−80 °C) and thawing (56 °C) cycle. After obtaining the DNA, five dilution points with base 5 were performed, which resulted in six points for the standard curve as follows: 5.10^3; 1.10^3; 2.10^2; 4.10; 8; and 1.6 oocysts/μL.

2.6. Molecular analysis

Samples' DNA was extracted by a DNeasy® PowerSoil® kit (QIAGEN, Hilden, Germany) as described previously. To identify the presence of total and viable (oo)cysts in sewage, nested-PCR for 18S rDNA genes of *Cryptosporidium* and *Giardia* was performed as described by Xiao *et al.* (1999) and Langkjaer *et al.* (2007), respectively (Table 1).

Table 1 | Oligonucleotides used in nPCR for the amplification of a fragment from the 18S rDNA gene region of *Cryptosporidium* spp. and *Giardia* spp. and in the qPCR for the 18S rDNA gene for *Cryptosporidium* in sewage, 2020

Molecular technique	Organism	Gene	Sequence	Amplicon	Reference
nPCR	*Cryptosporidium* spp.	18S rDNA	*First reaction* 5′-TTCTAGAGCTAATACATGCG-3’ 5′-CCCTAATCCTTCGAAACAGGA-3’	1,325 pb	Xiao *et al.* (1999)
			Second reaction 5′-GGAAGGGTTGTATTTATTAGATAAAG-3’ 5′-AAGGAGTAAGGAACAACCTCCA-3’	826–864 pb	
	Giardia spp.	18S rDNA	*First reaction* 5′-CATCCGGTCGATCCTGCC-3’ 5′-GTCGAACCCTGATTCTCCG-3’	292 pb	Langkjaer *et al.* (2007)
			Second reaction 5′-GACGCTCTCCCCAAGGAC-3’ 5′-CTGCGTCACGCTGCTCG-3’	130 pb	
qPCR	*Cryptosporidium* spp.	18S rDNA	*Primer forward* 5′-GTTTTCATTAATCAAGAACGAAAGTTAGG-3’ *Primer reverse* 5′-GAGTAAGGAACAACCTCCAATCTCTAG-3’ *Probe* 5′FAM-TCAGATACCGTCGTAGTCTTAACCATAAACTATGCC-3′ TAMRA	106 pb	Burnet *et al.* (2013)

Quantification of total and viable *Cryptosporidium* oocysts was performed by TaqMan-qPCR using StepOnePlus™ equipment (Applied Biosystems, Foster City, CA, USA). Oocysts were detected using forward and reverse primers and a probe described by Burnet *et al.* (2013) (Table 1).

The qPCR reaction for *Cryptosporidium* was composed of: 3 mM MgCl2 (MasterMix HOT FIREPol® qPCR Mix Plus ROX −15 mM MgCl2); 150 nM of the forward and reverse primers; 250 nM of the FAM-TAMRA probe; 12.9 μL of ultrapure water and 2 μL of sample DNA, with a total volume of 20 μL. The DNA amplification cycles were: initial denaturation at 95 °C for 10 min; 50 cycles of denaturation at 95 °C for 15 s and annealing combined with amplification at 60 °C for 60 s. The absolute quantification was performed by comparing the duplicate reactions of the samples and the dilutions in base 5 of the standard curve described previously.

2.7. Quantification of oocysts in sewage

To quantify the total number of oocysts recovered within the sewage sample (*oAq*), the total oocysts that had their DNA extracted (*oq* and *ve*) were divided by the total volumes obtained in each of the stages of purification and diagnosis (*vaq*, *vEs* and *vP*). Equation (1) is expressed as:

$$oAq=\{oq[(ve/vaq)(vP/vEs)]/20\}$$

where *oAq* is the total number of oocysts recovered per sample, *oq* is the number of oocysts obtained by the absolute quantification of qPCR, *ve* is the total volume obtained in DNA extraction (μL), *vaq* is the volume of DNA used in the qPCR, *vP* is the total volume obtained from the purification, *vEs* is the volume of purified sample used for DNA extraction, and 20 is the amount of 18S gene copies in one oocyst.

The concentration of oocysts/L in the sewage samples (*oWW*) was calculated by the total of oocysts recovered over the total volume of sewage used for concentration multiplied by one million. Equation (2) is expressed as: $oWW=(oAq/vWW)1{,}000{,}000$ where oWW is the concentration of oocysts in the sewer/L and *vWW* is the total volume of sewage used for concentration (μL).

2.8. Statistical analysis

Descriptive and inferential statistical analysis was performed using the R software (R Foundation, Vienna, Austria). The following tests were performed: normality test (Shapiro–Wilk); the homogeneity of variance test (*F*-test); *t*-test for two

samples; Wilcoxon unpaired and paired test for two samples; Spearman correlation and Simple Linear Regression. All tests had a significance level of 5%.

2.9. Spatial description

Through geoprocessing, using the QGIS software (QGIS.org Association – Free Software Foundation, Massachusetts, NY, USA), the quantification results of viable (oo)cysts in the affluent and effluent of the WWTP were related to a map showing the WWTP itself, the region from which the sewage was collected and the water body that has contact with the discharged effluent. The geographic layout of the locations contacting the receiving surface water, which receives the treated sewage, was obtained on the website of the Brazilian Institute of Geography and Statistics (IBGE) (Brazil 2019).

3. RESULTS AND DISCUSSION

Ten samples of raw- and treated-sewage were weekly obtained, which derived two aliquots per collection for raw sewage (RS: n=10; RS-PMA: n=10) and treated sewage (TS: n=10; TS-PMA: n=10). From these samples, with respect to the hydraulic detention time, seven times, it was possible to follow up on the sewage that arrives at the station after the treatment. According to nPCR, the viable (oo)cysts of *Cryptosporidium* and *Giardia* occurred in 100% (10/10) of the raw sewage samples. In the treated sewage, the frequency of *Cryptosporidium* was 100% (10/10) and *Giardia* was 70% (7/10), in which viable (oo)cysts were detected in 60% (6/10) and 30% (3/10) of them, respectively. On average, 50% of the oocysts counted in the WWTP affluent were viable and, after treatment, 6.34% of them remained viable.

The occurrence of *Cryptosporidium* and *Giardia* in raw sewage samples indicates that both protozoa are shed by the population that WWTP covers. *Cryptosporidium* quantification revealed that a high oocysts' concentration (average: 908.2 oocysts/L; SD: 341.45) reaches the WWTP. Both protozoa were found frequently in raw sewage worldwide, including in Brazil. However, the frequency of parasites and the concentration of oocysts presented here were not expected considering the characteristics of locality and population (Ramo *et al.* 2017; Xiao *et al.* 2018).

In agreement with this higher occurrence than expected, some locality parameters can be highlighted as a middle population density (161.88 hab/km²) under an agricultural system; a regular HDI (0,726); a high sanitation covered (96% of sewage caught and drinking water distributed); and a low hospitalization per diarrhea (1.7/1,000 inhabitants) (Brasil 2019). Worldwide, other studies have shown a range of 0–6,000 oocysts/L of *Cryptosporidium* and 10–12,225 cysts/L of *Giardia* (Santos *et al.* 2011; Taran-Benshoshan *et al.* 2015; Ramo *et al.* 2017; Xiao *et al.* 2018). Although an accurate comparison is not possible, because of the different detection methodologies and site descriptions (Rousseau *et al.* 2018), the highest amount of oocysts was counted in Campinas (6,000 oocysts/L), a big industrial city in Brazil (Santos *et al.* 2011), and the next highest was counted in Wuzhi (197 oocysts/L), a middle-size agricultural city in China (Xiao *et al.* 2018). According to this scenario, apparently lower quantities of oocysts are expected in sewage as the population density and anthropogenic activities decrease.

As frequencies of parasites and the amount of oocysts in this study were higher for an urban population, they may be explained by rural activities. The economic activity of the locality is primarily agro-industrial, composed of micro and medium industries which is a common social structure in Southern Brazil. Therefore, it might be possible that the illegal dumping of agro-industries occurred in domestic sewage, a hypothesis which is emphasized by the high prevalence of *Cryptosporidium* in cattle not only in the region but also in the whole country (Toledo *et al.* 2017). These illegal discharges, beyond impairing the efficiency of the treatment system still compromise the characterization of the population's health, causing misunderstandings in sewage-based epidemiology assessments (de Medeiros *et al.* 2017).

Moreover, the probable saturation of the sewage treatment system may complicate the elimination of these protozoa. This could be seen in the viable forms of parasites detected in the treated sewage, with high frequencies of protozoa (60% *Cryptosporidium* and 30% *Giardia*) and a high concentration of *Cryptosporidium* (average: 92.8 oocysts/L; SD: 53.42 oocysts/L). Viability measurements are essential to understand disease risks for populations that can be caused by the transmission through environments contaminated by sewage. The importance of this viability assessment can be seen especially in the case of such protozoa that, besides the low infectious dose, have infectious forms highly resistant to treatments commonly applied to water and sewage (Baldursson & Karanis 2011; Troeger *et al.* 2018).

Cryptosporidium viable forms presented twice the frequency of *Giardia* in treated sewage. A greater elimination of *Giardia* in wastewater samples compared to *Cryptosporidium* was already reported (Taran-Benshoshan *et al.* 2015; Ramo *et al.* 2017). Therefore, the difference in elimination possibly occurred due to the greater resistance of oocysts to the treatment. Even with

this difference, both protozoa are mostly found in wastewater compared to other water matrices and were detected in treated effluents from WWTPs (Nguyen *et al.* 2016). This alerts us to their potential of reaching water bodies in concentrations that represent risks to human and animal health (Xiao *et al.* 2018). This potential is evidenced by waterborne outbreaks caused by protozoa in which *Cryptosporidium* is the main etiology, followed by *Giardia* (Baldursson & Karanis 2011). Considering the discharge and consequent contamination of water bodies with effluents from WWTPs containing parasites, sewage can play a role as an environmental reservoir of these protozoa and may contribute to the maintenance of the endemicity of these parasites in the population.

The concentration of *Cryptosporidium* in the raw sewage was on an average 3.7 times lower than that in the treated sewage (Table 2 and Figure 2). The quantifications of oocysts in TS did not present a normal distribution (Shapiro–Wilk: *p*-value 0.001), which was evidenced by the high standard-deviation (SD: 3,948.86 oocysts/L). This higher amount of total

Table 2 | Frequency of total and viable (oo)cysts of *Cryptosporidium* and *Giardia* with average concentrations of *Cryptosporidium* in an affluent and effluent from a WWTP in Southern Brazil, 2020

	Giardia	***Cryptosporidium***									
	nPCR		**nPCR**		**qPCR**		**Oocysts/L**				
Aliquot	**%**	**(*n*/*N*)**	**%**	**(*n*/*N*)**	**%**	**(*n*/*N*)**	**Mean**	**Confidence interval 95%**		**Min**	**Max**
RS	100	(10/10)	100	(10/10)	100	(10/10)	908.2	242.4	1,574.0	452.2	1,456.5
RS-PMA	100	(10/10)	100	(10/10)	100	(10/10)	438.5	−10.9	887.9	151.0	849.2
TS	70	(07/10)	100	(10/10)	100	(10/10)	3,323.8	−4,376.5	11,024.1	306.1	13,492.0
TS-PMA	30	(03/10)	60	(06/10)	100	(10/10)	92.8	−11.4	196.9	29.88	184.36

Note: RS, raw sewage without PMA; RS-PMA, raw sewage with PMA; TS, treated sewage without PMA; TS-PMA, treated sewage with PMA; PMA, propidium-monoazide dye.

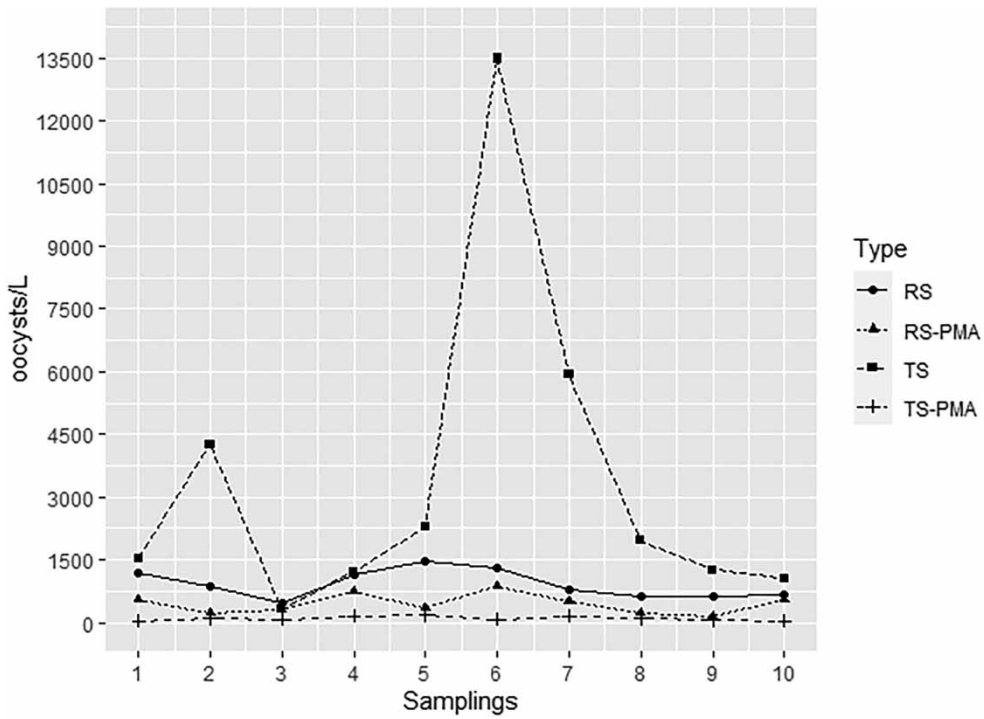


Figure 2 | Concentration of total and viable *Cryptosporidium* oocysts in an affluent and effluent from a WWTP, in 10 weekly samplings from September to November 2020, Southern Brazil. *Note:* Type (Type of aliquot); RS (Raw sewage without PMA); RS-PMA (Raw sewage with PMA); TS (Treated sewage without PMA); TS-PMA (Treated sewage with PMA); and PMA (Propidium-monoazide dye).

Cryptosporidium oocysts (viable and non-viable) in the TS was not an expected result (Ramo *et al.* 2017; Xiao *et al.* 2018). Results demonstrated less variation in the parasite's concentration in raw sewage compared to the variation in the effluent of the WWTP. This higher concentration might have occurred due to the environmental influence, since the treatment system consists of stabilization ponds, and due to the difference in the composition of the affluent and effluent (Nasser 2016; Devault *et al.* 2017). The stabilization ponds receive a 12-h daily solar incidence, so the higher oocyst concentration may have occurred by water evaporation. This fact is associated with lower PCR-inhibitors levels in treated sewage, and a higher concentration of available target DNA, which allowed this greater quantification in this sample by qPCR.

For aliquots with PMA, the viable oocysts amount in the raw sewage (RS-PMA) was 5.6 times higher than that in the treated sewage (TS-PMA) (Table 2 and Figure 2). In all seven raw and treated sewage follow-ups, with respect to the detention time, raw sewage with PMA (RS-PMA) had a higher concentration of oocysts than treated sewage stained with PMA (TS-PMA) (Table 2). The consistent results in lower concentrations of viable oocysts in the treated sewage when compared to the raw sewage demonstrate the applicability of the adapted methodology for detecting viability. Therefore, both concentration and purification methods efficiently allowed the detection of (oo)cysts by nPCR and qPCR.

Comparing the two methods tested for *Cryptosporidium* viability assessment, PMA-nPCR presented 20% (4/20) of negative results, observed in the treated sewage samples, differently from PMA-qPCR, which did not present a negative result. It indicates that qPCR is more sensitive to this protozoan surveillance in sewage matrices. In this sense, PMA-qPCR, associated with the concentration and purification techniques employed in this study, proved to be a sound methodology for quantifying viable *Cryptosporidium* oocysts in sewage matrices even when a low budget is available (Ma *et al.* 2016). However, with this methodology, some limitations must be considered in aiming to minimize the diagnostic errors. These are related to (i) the sewage treatments that make (oo)cysts non-viable without compromising their cell membranes (e.g., UV on the surface of the WWTP stabilization ponds); (ii) the high concentration of particles that may hinder PMA binding to dsDNA target (e.g., non-target dsDNA); and (iii) the solutions with high levels of turbidity, which can limit the photoactivation of the dye (Liang & Keeley 2012).

In this study, both the presence of other microorganisms and the level of turbidity were reduced using the specific concentration methodology for protozoa, associating centrifugation, filtration and purification in sucrose solution. Nevertheless, the non-viable oocysts with intact walls may have been diagnosed as viable due to the impossibility of this proposed methodology to correct this error (Liang & Keeley 2012). About the turbidity level, the averages of raw and treated sewage were statistically different (*t*-test – *p*-value: 0.00004). The average observed in raw sewage was 332 NTU (CI 0.95: 221.55–442.45), and in treated sewage, it was 75.25 NTU (CI 0.95: 56.7–93.8). There was a low positive correlation between oocyst concentration and turbidity for raw sewage (simple linear regression: $Y=125.44+0.01*NTU$; $R^2=0.4312$). For the treated sewage, there was no correlation with the statistical significance (Spearman's correlation – *p*-value: 0.89).

With respect to the 14-day hydraulic detention time for the seven follow-ups, it was possible to verify the statistical significance of the treatment for removing viable oocysts (paired Wilcoxon Test – *p*-value: 0.01). The maximum removal value of viable oocysts found was 1.02 and the persistence of viable oocysts which reached the receiving waterbody was an average of 31.17% (Table 3). The average concentration of oocysts inactivated by the sewage treatment was 349.95 (CI 0.95: −181.49; 881.38) (Table 2).

Table 3 | *Cryptosporidium* oocysts concentration according to detention time, in affluent and effluent of a WWTP, related to the removal and persistence of oocysts after treatment, Ibiporã, Paraná, BR, 2020

Samplings (raw and treated)	Monitoring (detention period: 14 days)	Viable oocysts/L		Removal value (log)	Persistence (%)
		Raw	Treated		
1 and 3	1	525.09	74.04	0.85	14.10
2 and 4	2	224.58	148.95	0.18	66.32
3 and 5	3	314.27	184.36	0.23	58.66
4 and 6	4	733.63	70.39	1.02	9.59
5 and 7	5	336.19	149.77	0.35	44.55
6 and 8	6	849.23	97.17	0.94	11.44
8 and 10	7	221.18	29.88	0.87	13.51

Variations in the removal and persistence of viable forms of both *Cryptosporidium* and *Giardia* highlight the need to monitor the viability of these protozoa in effluent from WWTPs. Inferential models are used to estimate the population risk of infection, based on the oocyst quantity in raw sewage. However, this may be subject to unforeseen errors due to the bias on real concentrations estimation on treated sewage (Xiao *et al.* 2018). Thus, it is important to characterize the variations in the concentration of (oo)cysts in the arrival and departure of sewage, for the establishment of more accurate predictive models that can be used in sewage-based epidemiology, when there are similar social and health contexts among regions (Xiao *et al.* 2018; Mao *et al.* 2020). In this study, the greater persistence of viable *Cryptosporidium* oocysts (60% of TS-PMA samples), compared to *Giardia* (30% of TS-PMA samples), indicates that the treatment by stabilization ponds with a 14-day detention is more efficient for removing *Giardia*. Greater removal of oocysts was reported in activated sludge than that in the stabilization ponds' systems, although at least 20 days of detention are required for achieving effectiveness in the removal of protozoa when stabilization ponds treatment is applied (Taran-Benshoshan *et al.* 2015; Nasser 2016). This parameter corroborates with the results obtained in this study, which showed an average persistence of 92.8 oocysts/L.

The Jacutinga River received an average of 3,345.44 oocysts/s (SD: 1,925.92 oocysts/s) during the 10 weeks of study (annual flow average of WWTP effluent: 129.78 m^3/h). For the upstream of the sewage point of discharge of the viable forms of *Cryptosporidium* and *Giardia*, there has been located the water catchment point of Ibiporã city, which is downstream of the middle-size city of Londrina. The course of the river downstream of the WWTP discharge flows through a region of agricultural activity, with no urban perimeter regions until its discharge on the Tibagi River (Figure 3).

It was possible to verify that the Jacutinga River receives viable forms of the parasites in the discharge of WWTP effluent. The presence of these parasites in the water body can pose a risk to animals and humans that are supplied by the river (Toledo

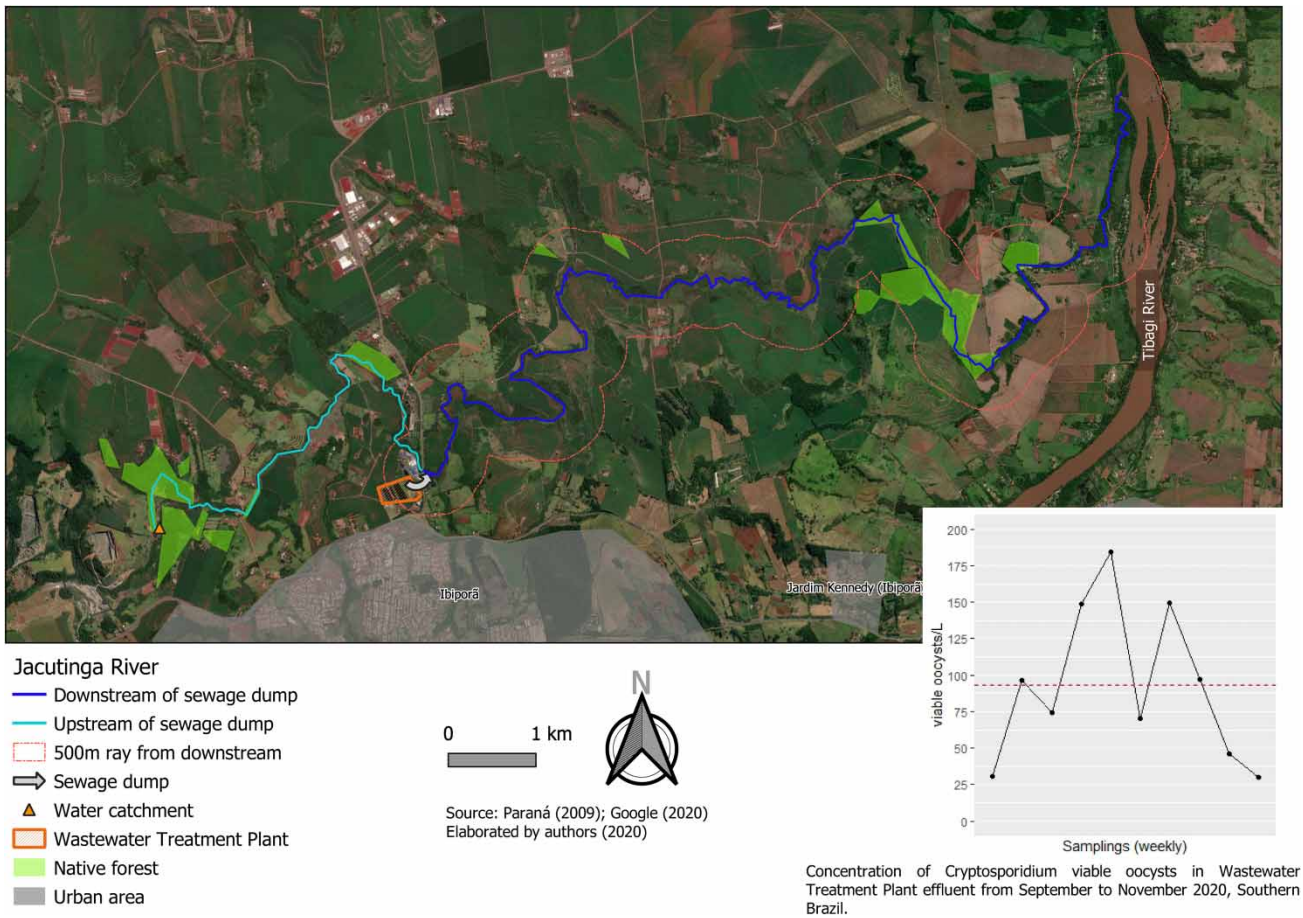


Figure 3 | Satellite view of the study region with the representation of the Jacutinga River upstream and downstream of the effluent discharge site of the wastewater treatment plant with the concentration of viable *Cryptosporidium* oocysts, Ibiporã, Paraná, Brazil, 2020.

et al. 2017). In this region, the main concern is the use of this water for the irrigation of crops and animal watering, considering the predominance of agricultural activities in the locality. To avoid or reduce the concentrations of protozoa dumped into the river, it is necessary to associate: (i) the reduction of parasites that reach the WWTP, by screening and preventing possible illegal discharges and (ii) improving the treatment to remove these resistant protozoa, starting with the increasing detention time to 20 days.

4. CONCLUSIONS

Cryptosporidium and *Giardia* presented endemic characteristics in the region that supplies the studied WWTP which can be influenced by agro-industrial dumps. The (oo)cysts of these protozoa arrive viable through the raw sewage and remain viable after WWTP treatment. *Cryptosporidium* is more resistant to stabilization ponds than *Giardia*. The higher occurrence and concentration of parasites in all samples without PMA compared to samples with the dye demonstrates that the methodology is able to quantify viability in sewage matrices, even under high turbidity.

Viable *Cryptosporidium* oocysts arrive in high concentrations at the WWTP. Even with the reduced concentration by sewage treatment, these oocysts reach the receiving water body in high amounts. Despite the low number of follow-ups of raw and treated sewage, the variability between the persistence values highlights the need for monitoring these parasites in sewage effluent, to avoid losses regarding human, animal and environmental health.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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