CrossMark

Performance comparison of three methods for detection of *Giardia* spp. cysts and *Cryptosporidium* spp. oocysts in drinking-water treatment sludge

Guilherme Lelis Giglio · Lyda Patricia Sabogal-Paz

Abstract Detecting pathogenic protozoa in drinking-water treatment sludge is a challenge as existing methods are complex, and unfortunately, there are no specific technical standards to follow. Selecting an efficient analytical method is imperative in developing countries, such as Brazil, in order to evaluate the risk of parasite infection. In this context, three methods to detect *Giardia* spp. cysts and *Cryptosporidium* spp. oocysts were tested in sludge generated when water with protozoa and high turbidity was treated. Jar testing was carried out using polyaluminium chloride as a coagulant to generate the residue to be analyzed. The results showed that calcium carbonate flocculation with reduced centrifugation and immunomagnetic separation obtained the highest recoveries in the tested matrix showing $60.2\% \pm 26.2$ for oocysts and $46.1\% \pm 5$ for cysts. The other two methods, the first using the ICN $7\times$ cleaning solution and the second considering the acidification of the sample, both followed by the immunomagnetic separation step, also presented high recoveries showing $41.2\% \pm 43.3$ and $37.9\% \pm 52.9$ for oocysts and $11.5\% \pm 85.5$ and $26\% \pm 16.3$ for cysts, respectively. Evidently, these methods and others should be studied in order to make it possible to detect protozoa in settled residue.

Introduction

Protozoa outbreaks worldwide

The emergence of various water treatment technologies does not ensure that waterborne diseases are eradicated, as there are still reports of outbreaks related to water drinking supply systems, involving *Giardia* and *Cryptosporidium* protozoa, even in developed countries (Karanis et al. 1998; Baldursson and Karanis 2011; Efstratiou et al. 2017a). As reported by Efstratiou et al. (2017a), at least 381 outbreaks attributed to parasitic protozoa were documented from January 2011 to December 2016 and 99% of these cases were described in New Zealand, North America, and Europe. According to these authors, less-developed countries are probably the most affected by such diseases, and consequently, installing surveillance systems in these nations is essential for improving the health of the population. However, the costs involved and the difficult protozoa detection methods available are a limitation in developing countries.

Protozoa detection in developing countries

In Latin American countries, waterborne diseases are frequent, and in this scenario, *Giardia* and

G. L. Giglio · L. P. Sabogal-Paz (✉)
Department of Hydraulics and Sanitation, São Carlos School of Engineering, University of São Paulo, 400 Trabalhador São-carlense Avenue, São Carlos, São Paulo 13566-590, Brazil
e-mail: lysaboga@sc.usp.br

Cryptosporidium protozoa can be included. Rosado-García et al. (2017) identified that only 16 outbreaks of waterborne-protozoa were reported in these countries from 1979 to 2015. According to these authors, Latin American countries do not have a coherent methodology to detect protozoa in water samples. It is essential that less-developed countries can detect protozoan in environmental matrices to establish effective and suitable diagnoses of health risks. Obviously, detection methods must take into account the economic availability and particular needs of each country.

Giardia cysts and *Cryptosporidium* oocysts in environmental matrices

Giardia spp. cysts and *Cryptosporidium* spp. oocysts are found in the environment in their resistance forms (cysts and oocysts, respectively) and can cause infections in humans. The main symptom is bouts of diarrhea. They need a host to complete their life cycles. However, Koh et al. (2013) demonstrated that the *Cryptosporidium* genus may be able to reproduce outside the host (aquatic biofilms) causing new implications in the public health area.

These protozoa can survive for a long time in aquatic environments (Olson et al. 1999), they can pass through Water Treatment Plant (WTP) filters due to the reduced size (4 to 18 μm), and they are resistant to the action of disinfectants commonly used in WTPs. According to Walker et al. (1998), laboratory and field studies have indicated that *Cryptosporidium* oocysts can survive for months in the soil in cold and dark conditions and up to a year in water with low turbidity. Although water treatment technologies are effective in removing *Giardia* and *Cryptosporidium*, these protozoa can be found in drinking water, when there are treatment deficiencies.

Although these protozoa can be removed from drinking water, viable and potentially infectious *Cryptosporidium* oocysts may remain in the residue generated after treatment (Keegan et al. 2008). Karanis et al. (1996) detected *Giardia* cysts and *Cryptosporidium* oocysts in filter backwash water, and they warned of the hazard of recycling this residue in WTPs.

In countries such as Brazil, 67% of WTPs discarded their sludge directly into rivers or sea and 26% into land or landfills, according to data from 2008 (IBGE Instituto Brasileiro de Geografia e Estatística (Brazilian Institute of Geography and Statistics) 2010). These residues can contain viable cysts and oocysts, which are potentially infectious and persistent in aquatic environments. Andreoli and Sabogal-Paz (2017) showed that approximately 18% of *Giardia* spp. cysts and 82% of *Cryptosporidium* spp. oocysts may be retained in WTP sludge when dissolved air flotation (DAF) jar testing was performed. It should be mentioned that these protozoa were detected in Brazilian supply sources (Hachich et al. 2004; Sato et al. 2013); therefore, these parasites may appear in the WTP sludge causing potential health risks.

Dumping WTP sludge into supply sources is prohibited by Brazilian legislation. However, as environmental agencies do not always inspect the water sources and sludge treatment is costly, this illegal practice often takes place. Clearly, adequate residue management can eliminate health risks and avoid a detailed characterization of these complex environmental matrices.

Giardia cyst and *Cryptosporidium* oocyst detection in complex matrices

The threat of *Giardia* and *Cryptosporidium* species in WTP residues poses a challenge in terms of detection, as a standard protocol does not exist. Method 1623.1 from the United States Environmental Protection Agency (USEPA 2012) is validated exclusively for detecting protozoa in water.

Reagents, materials, and equipment used in the various methods available to detect cysts and oocysts in environmental samples incur high costs and are technically and analytically complex (Maciel and Sabogal-Paz 2016; Andreoli and Sabogal-Paz 2017). Using a simplified protocol that can detect *Giardia* cysts and *Cryptosporidium* oocysts in a regional context will be able to evaluate the dynamics of these protozoa in the residues aiming at their subsequent treatment.

Concerning detection of protozoa, filtration methods are the most popular for isolating parasites from water samples. Nevertheless, they have limitations on some matrices due to filter clogging. Options for monitoring protozoa can be flocculation-sedimentation procedures (Efstratiou et al. 2017b). Some methods for detecting protozoa in WTP residues have been described in Keegan et al. (2008) and Andreoli and Sabogal-Paz (2017), involving acidification of the sample and calcium carbonate flocculation (CCF), respectively, both with purification by immunomagnetic separation (IMS).

CCF developed by Vesey et al. (1993) has been used to detect protozoa especially in samples with high

turbidity (Andreoli and Sabogal-Paz 2017; Feng et al. 2011), and ICN 7× cleaning solution was used most to detect helminth eggs in environmental samples (Steinbaum et al. 2017). However, De Oliveira (2012) was successful when centrifugation and ICN 7× cleaning solution were used in soil samples to recover *Cryptosporidium* spp. oocysts and *Giardia* spp. cysts.

Detection of pathogenic protozoa is common in wastewater treatment plant (WWTP) residues to evaluate the health risk of disposing of them in agricultural soil. These biosolids play an important role in the epidemiology of giardiasis and cryptosporidiosis; however, there is no standardized methodology to detect them (cysts and oocysts) in these complex matrices (Sidhu and Toze 2009). It can be observed that few studies have evaluated these parasites in WTP sludge (Karanis et al. 1996; Keegan et al. 2008; Maciel and Sabogal-Paz 2016; Andreoli and Sabogal-Paz 2017).

Research objectives

Considering the lack of research on pathogenic protozoa detection in WTP residues and the existing problems in countries such as Brazil, this paper considered the performance of three methods for detection of *Giardia* spp. cysts and *Cryptosporidium* spp. oocysts in settled residue generated after treating drinking water with high turbidity. Treatability tests in jar tests were carried out using polyaluminium chloride (PACl) as a coagulant. In this study, the protocols described in Vesey et al. (1993) with CCF, Keegan et al. (2008) with acidification of the sample, and De Oliveira (2012) with ICN 7× cleaning solution were tested, all involving reduced centrifugation and purification by IMS. The costs of the methods were analyzed, and the best method for the matrix tested was pointed out according to specific statistical tests.

Materials and methods

The research was divided into two stages. Step A consisted of preparing the water from the study by adding kaolinite to well water (without the presence of protozoa) at a ratio of 0.16 g L^{-1} required to obtain a turbidity of approximately 130 NTU. This water was prepared in order to eliminate possible interferences inherent to natural samples, as the purpose of the research was to evaluate the performance of the three selected purification methods.

After preparing the water from the study, treatability assays in jar tests were performed to optimize the parameters associated to the treatment. The coagulation diagrams were constructed to select the optimal points (coagulant dosage versus coagulation pH, with and without pH correction). Then, rapid mixing, slow mixing, and sedimentation velocity parameters were optimized. PACl with Al$_2$O$_3$ content of 16.36% was used in the tests. After finalizing the treatability tests, new tests were carried out to characterize the settled residue. In Step A, the physical-chemical and microbiological analyses performed followed the procedures described in American Public Health Association – APHA, American Water Works Association – AWWA, and Water Environment Federation – WEF (2012).

Step B evaluated three purification methods of protozoan detection described in Vesey et al. (1993), Keegan et al. (2008), and De Oliveira (2012). The centrifugation step was performed as recommended in USEPA (2012). However, according to the protocol proposed by Vesey et al. (1993), the time was increased for 20 min. The assays were performed in triplicate under the same conditions. This means that for all the experiments, inoculum concentrations with cysts and oocysts were equally introduced into the jars before starting each assay, and the enumeration protozoa technique were not changed.

Recovery of the cysts and oocysts obtained was compared (as guidance) to the standard established by Method 1623.1 from USEPA (2012), as there is no specific technical standard to be followed for WTP residues. The three methods had seven common steps (step 1 through step 7). In addition, all vessels and materials that were exposed to the protozoa were previously washed with Tween 80 (0.1%).

Step 1 determined the number of target organisms to be inoculated in the water from the study. A concentration between 10^2 and 10^3 cysts L^{-1} and oocysts L^{-1} was established, which was inoculated directly into each jar of the jar test apparatus. These concentrations were selected because they were generally found in Brazilian supply sources (Hachich et al. 2004; Sato et al. 2013) and were likewise studied by Maciel and Sabogal-Paz (2016) and Andreoli and Sabogal-Paz (2017).

The volume of inoculum was determined by the arithmetic mean of protozoa counted in three identical aliquots taken after homogenization of the suspensions of *Cryptosporidium* spp. oocysts from Waterborne (USA) and *Giardia* spp. cysts purified at the

Protozoology Laboratory at the University of Campinas (Brazil). Counting was performed by fluorescence-isothiocyanate (FITC) microscopy using 4′,6-diamidino-2-phenylindole (DAPI) and differential interference contrast (DIC).

In step 2, cyst and oocyst inoculums were added to the water from the study in the jars and jar testing procedures were carried out using the parameters optimized in Step A. After the procedure described, the settled residue was generated.

In step 3, the generated sample was transferred equally to three 50-mL Falcon® tubes. The jars were washed with Tween 80 (0.1%), and the resulting liquid was poured into each Falcon® tube. The tubes were centrifuged at 1500g for 20 min for the protocol from Vesey et al. (1993) and 1500g for 15 min in the remaining protocols. Only one tube was randomly selected to the next phase, and the other two tubes were discarded. It is important to note that each tube generated 0.5 mL of pellet—maximum volume recommended by USEPA (2012) for processing via IMS. The aim of this procedure was to reduce the high costs incurred by the test taking into account the economic resources available in Latin American countries, such as Brazil. To compensate for the loss of sample, and hence protozoa, the amount of cysts and oocysts counted on the microscope slide was increased by a multiplication factor equal to three (MF = 3) in Eq. 1.

$$R = \frac{(C1 + C2) \times MF}{\text{Inoculated protozoa}} \times 100\% \qquad (1)$$

where R is the recovery of cysts or oocysts according to the evaluated method (%), C1 is the cyst or oocyst counts in first acid dissociation, C2 is the cyst or oocyst counts in second acid dissociation, and MF is the multiplication factor.

In step 4, the concentrated sample, obtained after following the procedures described in Vesey et al. (1993), Keegan et al. (2008), and De Oliveira (2012), underwent the IMS procedure using the Dynabeads IDEXX® kit, according to the manufacturer's instructions. Two acid dissociations were performed in each sample. Therefore, 50 μL of each dissociation was transferred to two wells of the slide, totalling 100 μL.

Step 5 consisted of preparing the microscope slide. Merifluor ® Meridian GC kit reagents and DAPI solution were added to each well containing the samples, as recommended by the manufacturers.

Step 6 consisted of counting and enumerating the parasites under a microscope increased by × 200 to × 800. Microscopic visualization was carried out using FITC, DAPI, and DIC. Finally, in step 7, the recovery percentage of each method was calculated, according to Eq. 1.

The CCF method described in Vesey et al. (1993) was evaluated followed by IMS. In this case, after performing steps 1 to 2, the residue sample was homogenized using a magnetic stirrer for 20 min, and while stirring, 10 mL of 1.0 M calcium chloride ($CaCl_2$) and 10 mL of 1.0 M sodium bicarbonate ($NaHCO_3$) were added to the sample, and then the pH was adjusted to 10, dropping 5.0 M sodium hydroxide (NaOH). The sample was allowed to rest at room temperature overnight. The next day the supernatant was discarded, leaving only 100 mL in the jar. This sample was homogenized using a magnetic stirrer for 10 min. After that period, 20 mL of sulfamic acid (10%) was added. The mixture was stirred for another 5 min, and then step 3 was performed. The pH of the centrifuged sample was corrected to neutrality by adding Dulbecco's phosphate-buffered saline (DPBS) aliquots. Afterwards, a further centrifugation was carried out. A total of 0.5-mL pellet was obtained and the concentrated sample was processed following steps 4 to 7, described previously.

The protocol proposed by De Oliveira (2012) was also evaluated followed by IMS. Steps 1 to 3 were carried out. In this case, 5 mL of the centrifuged sample was homogenized and transferred to the flat-sided sample tube (FST) and was washed twice in 1.0 mL of ultrapure water. In addition, 5 mL of 1.0% MP BIO® cleaning solution (ICN 7×) was added to the FST in order to help disaggregate the cysts and oocysts of the soils found in the sample. Then, 12 mL of the blend was homogenized in a rotary mixer for 1.0 h, before the purification step was initiated via IMS. Afterwards, steps 4 through 7 were followed. The method did not involve pH changes in the sample; therefore, there was no need to correct the parameter.

The method described in Keegan et al. (2008) was also evaluated, followed by IMS. After following steps 1 to 3, the supernatant was discarded until the 10 mL mark and 0.015 N of sulfuric acid was added drop by drop until the pH was equal to 3. The sample was homogenized using a Pasteur pipette and centrifuged again. The pellet supernatant was discarded again and the sample received DPBS until the pH was neutralized (the procedure had additional centrifugations). The

5.0 mL volume containing 0.5 mL of the pellet was homogenized and transferred to the FST to start the IMS step, and then steps 4 to 7 were followed.

The analytical quality assays of the tested methods were not carried out because the residues are generated after the water treatment and the distributions of the cysts and oocysts occurs in a distinct way in all the phases of the conventional treatment (liquid and solid), according to Maciel and Sabogal-Paz (2016) and Andreoli and Sabogal-Paz (2017). Therefore, the number of cysts and oocysts found in the evaluated matrix (settled residue) is not known.

In order to verify the existence of statistical differences between the tested methods, F test for the ANOVA table with a significance level of 5% was used. Afterwards, the methods were compared using the Tukey test, also with a 5% level of significance.

This study equally assessed the costs of the main materials used in each protocol (i.e., Merifluor® and Dynabeads® kits). The quotes were updated to July 1, 2018, using the General Market Price Index at *Fundação Getúlio Vargas* (FGV), which is an index applicable to Brazil.

Results and discussion

Table 1 presents the optimum conditions of the water treatment that generated the settled residue studied.

Characteristics of water from the study Total alkalinity = 9.6 mg $CaCO_3$ L^{-1}; total aluminum = 1.32 mg Al L^{-1}; total coliforms = 6 CFU 100 mL^{-1}; *Escherichia coli* = absent; electrical conductivity = 52.9 μS cm^{-1};

hardness = 22.4 mg $CaCO_3$ L^{-1}; total iron = 0.32 mg Fe L^{-1}; total manganese = 0.017 mg Mn L^{-1}; zeta potential = − 22 mV; and turbidity = 133 NTU.

Characteristics of filtered water Total alkalinity = 20 mg $CaCO_3$ L^{-1}; total aluminum < 0.001 mg L^{-1}; total coliforms = absent; *Escherichia coli* = absent; electrical conductivity = 69.8 μS cm^{-1}; hardness = 15.0 mg $CaCO_3$ L^{-1}; total iron < 0.005 mg Fe L^{-1}; total manganese < 0.005 mg Mn L^{-1}; and turbidity = 0.18 NTU.

The settled residue obtained from the jar test presented the following characteristics: sedimented solids (mL L^{-1}) = 20 ± 1, total solids (mg L^{-1}) = 2916 ± 13, turbidity (NTU) = 3226 ± 187, and pH = 7.15 ± 0.1. These solids and turbidity values pose as a challenge in terms of detecting *Giardia* spp. cysts and *Cryptosporidium* spp. oocysts in WTP residues. According to Franco et al. (2012), high turbidity may influence IMS, as this step captures free cysts and oocysts, thus not adhering to sediments or other particles (e.g., flocs).

The higher the precipitate obtained in the concentration step, the lower the IMS efficiency, and this fact has limited the sample pellet by up to 0.5 ml in Method 1623.1 from USEPA (2012). Still regarding turbidity, the recovery of *C. parvum* oocysts can be drastically reduced with increasing turbidity of the matrix (Ochiai et al. 2005; Chang et al. 2007). On the other hand, the value of pH close to neutrality is favorable to the IMS procedure (USEPA 2012).

By adopting the methods described in Vesey et al. (1993), Keegan et al. (2008) and De Oliveira (2012) followed by IMS, mean recovery levels of *Giardia* spp. Cysts, and *Cryptosporidium* spp. oocysts were within the criteria described in Method 1623.1 (USEPA 2012). However, the coefficient of variation was high, except for the method proposed by Vesey et al. (1993), as in Table 2. The same number of cysts and oocysts was inoculated in the jar test (mean ± standard deviation) for Keegan et al. (2008) and De Oliveira (2012) because the assays were performed at the same time.

Cysts and oocysts were lost throughout the procedures (Table 2), a situation also described by Andreoli and Sabogal-Paz (2017). Turbidity in samples may have reduced the interaction between magnetic microspheres and protozoa in the IMS procedure (USEPA 2012). Likewise, there was a fluorescence reduction for cysts and oocysts during microscopic analysis, a phenomenon that makes visualization difficult.

Table 1 Parameters obtained from the jar test procedures

Parameter	No adjustment of pH
PACl dosage (mg L^{-1})	25
Al^{3+} concentration (mg L^{-1})	2.2
pH coagulation	6.7
Mean velocity gradient in fast mixing (s^{-1})	1000 (500 rpm)
Rapid mixing time (s)	10
Mean velocity gradient for flocculation (s^{-1})	25 (35 rpm)
Flocculation time (min)	30
Sedimentation velocity (cm min^{-1})	1.5

Table 2 Percentages of recovery of protozoa obtained by the methods tested

Methods (triplicate assays)	Cysts and oocysts inoculated in the jar (mean ± standard deviation)		Cysts and oocysts counted on the microscope slides		Recovery in relation to the inoculum (%) (Eq. 1)		Mean recovery (%)		Coefficient of variation (CV)	
	C	G	C	G	C	G	C	G	C	G
Vesey et al. (1993)	859 ± 58	1356 ± 20	141	208	49.2	46.0	60.2	46.1	26.2	5.0
			224	198	78.2	43.8				
			152	219	53.1	48.5				
Keegan et al. (2008)	741 ± 194	619 ± 87	148	61	59.9	29.6	37.9	26.0	52.9	16.3
			51	44	20.6	21.3				
			82	56	33.2	27.1				
De Oliveira (2012)	741 ± 194	619 ± 87	136	13	55.1	6.3	41.2	11.5	43.3	85.5
			117	47	47.4	22.8				
			52	11	21.1	5.3				
Method 1623.1 (USEPA 2012)							32–100	8–100	≤37	≤39

C Cryptosporidium spp., *G* Giardia spp.

When analyzing the samples through a microscope, impurities in the slide well were observed (Fig. 1) and these were larger in the protocols from Keegan et al. (2008) and Vesey et al. (1993), although the purification step via IMS was performed.

The protocol proposed by De Oliveira (2012) showed a better visualization of the target organisms (Fig. 1c); therefore, using ICN 7× cleaning solution at 1.0% helped disaggregate the cysts and oocysts of the soils found in the sample. Applying the cited protocol is also advantageous, as it does not alter the pH of the sample, favoring the maintenance of the viability of the cysts and oocysts, which enables animal infectivity testing when necessary.

For the method proposed by Vesey et al. (1993), the sample with high turbidity (settled residue) was processed, and it met the standard established by Method 1623.1 (Table 2). However, according to Franco et al. (2012), the method presents the following restrictions: (i) Variations in the reagent concentration and pH may decrease the recovery of cysts and oocysts, and there is also a loss of target organisms in the supernatant discarded in the procedure; (ii) the reagents used can cause a reduction in the fluorescence in the organisms and an increase in the residual fluorescence. Moreover, the concentrated sample has particulate material that makes it difficult to visualize the cysts and oocysts; therefore, purification via IMS is indispensable; and (iii) the pH of 10, required by the procedure, as well as the waiting time (overnight) can cause morphological and viability alterations, impairing animal infectivity testing.

The method proposed by Keegan et al. (2008) entailed using sulfuric acid to loosen the *Cryptosporidium* spp. oocysts and *Giardia* spp. cysts of the flocs formed in the sample. Nevertheless, this protocol requires successive centrifugations in order to neutralize the pH to perform the IMS, and this fact can cause a loss of parasites during the procedure. In addition, the reduction in pH may affect viability and animal infectivity testing.

When evaluating the three protocols tested, the importance of performing two consecutive acid dissociations in IMS was clear (Table 3).

A higher number of *Cryptosporidium* spp. oocysts was observed in the first acid dissociation (72.6 to 92%) when compared to the second acid dissociation (8 to 27.4%). In the case of *Giardia* spp., a difference between dissociations was less significant. Only for the methods proposed by Keegan et al. (2008) and De Oliveira (2012), a higher number of cysts captured in the second dissociation can be observed with 52.9 and 55.2%, respectively.

According to Maciel and Sabogal-Paz (2016), the second acid dissociation was essential in terms of obtaining greater recoveries in the IMS. Increases in the detection of cysts and oocysts were obtained (53% ± 29 and 77% ± 43, respectively) when the second dissociation was added while evaluating the settled residue samples.
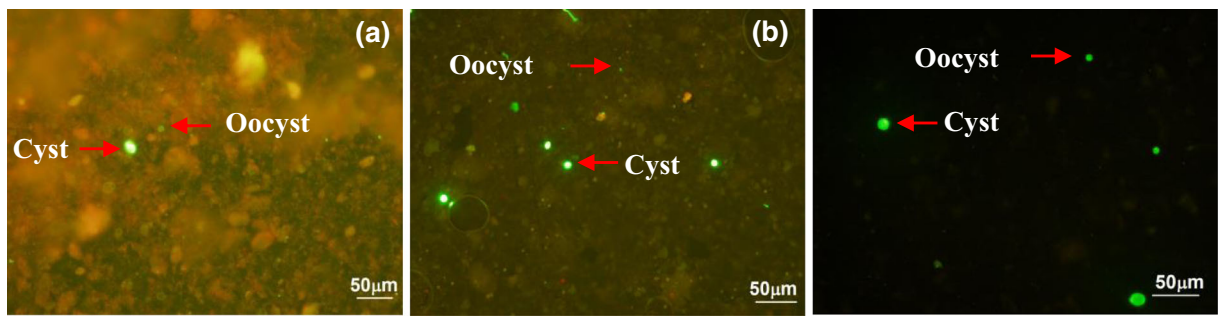
Fig. 1 Image of cysts and oocysts in the samples by direct reaction with monoclonal antibody conjugated with FITC (fluorescein isothiocyanate). **a** Method proposed by Vesey et al. (1993) in × 400. **b** Method proposed by Keegan et al. (2008) in × 200. **c** Method proposed by De Oliveira (2012) in × 400

De Oliveira (2012) observed that, on average, 51.5% of *Cryptosporidium* spp. oocysts and 39.3% of *Giardia* spp. cysts were recovered in the first acid dissociation, while in the second acid dissociation, only 5.7% of the oocysts and 11.1% of the cysts were seen. Despite this, the second dissociation boosted the efficiency of mean recoveries that were $57.2\% \pm 21.8$ for oocysts and $50.4\% \pm 28.2$ for cysts.

Chang et al. (2007) evaluated the influence of the number of acid dissociations applicable to IMS concerning the recovery of *C. parvum* oocysts in deionized water. The authors observed that from the third dissociation, the cumulative number of oocysts remained stable.

Karanis and Kimura (2002) and Campbell et al. (1994) indicated that methods involving pH changes in sample processing might reduce the infectivity of *C. parvum* oocysts. However, in our research, the main objective was to estimate the presence or absence of parasites and, consequently, the potential risk of the studied matrix in the context of developing countries.

When the *F* test was adopted by ANOVA followed by the Tukey test, it was observed that there was no significant statistical difference between the methods

Table 3 Comparison between the first and second acid dissociation in relation to the cysts and oocysts that were counted in the samples

Method	*Cryptosporidium* spp. oocysts observed under a microscope in absolute number and percentage					*Giardia* spp. cysts observed under a microscope in absolute number and percentage				
	1st	%	2nd	%	Total	1st	%	2nd	%	Total
Vesey et al. 1993	125	88.7	16	11.3	141	99	47.6	109	52.4	208
	213	95.1	11	4.9	224	108	54.5	90	45.5	198
	140	92.1	12	7.9	152	124	56.6	95	43.4	219
Mean	159.3	92.0	13.0	8.0	172.3	110.3	52.9	98.0	47.1	208.3
Standard deviation	47.1	3.2	2.6	3.2	45.1	12.7	4.7	9.8	4.7	10.5
Keegan et al. 2008	116	78.4	32	21.6	148	17	27.9	44	72.1	61
	43	84.3	8	15.7	51	24	54.5	20	45.5	44
	73	89	9	11	82	33	58.9	23	41.1	56
Mean	77.3	83.9	16.3	16.1	93.7	24.7	47.1	29.0	52.9	53.7
Standard deviation	36.7	5.3	13.6	5.3	49.5	8.0	16.8	13.1	16.8	8.7
De Oliveira (2012)	76	55.9	60	44.1	136	1	7.7	12	92.3	13
	106	90.6	11	9.4	117	34	72.3	13	27.7	47
	37	71.2	15	28.8	52	6	54.5	5	45.5	11
Mean	73.0	72.6	28,7	27.4	101.7	13.7	44.8	10.0	55.2	23.7
Standard deviation	34.6	17.4	27.2	17.4	44.0	17.8	33.4	4.4	33.4	20.2

1st first acid dissociation, *2nd* second acid dissociation

when the recoveries of *Cryptosporidium* spp. oocysts were analyzed. On the other hand, in the case of *Giardia* spp., a significant difference was observed in the recoveries for the method proposed by Vesey et al. (1993), and it was therefore higher in the recovery of cysts. CCF with IMS may be feasible to detect pathogenic protozoa in WTP residues. However, more research is needed to evaluate protozoa in this complex matrix.

The cost of the Merifluor® and Dynabeads® kits is shown in Table 4, and a high unit cost for the first kit can be observed (US$ 114.09 ± 8.71).

Regarding the application of these products, the three methods were performed under the same conditions. The Merifluor ® kit was used against the inoculum (three units per sample) and also to visualize the target organisms after applying the detection methods (two units per sample).

The Dynabeads® kit was applied once in each sample. It is important to highlight that all the tests were done in triplicate; therefore, the total costs for the methods tested are in Table 5. Other reagents used in the methods were inexpensive, and consequently, they were not included in the cost evaluation.

The total value for a single sample, including the costs for Merifluor and Dynabeads, was US$ 195.0 ± 78.3 in April 1, 2016, when CCF with IMS was applied by Andreoli and Sabogal-Paz (2017). These values were slightly higher than indicated in Table 4 (US$ 190.40 ± 8.10). Evidently, the difference in results depends on the currency exchange of dollar to real in Brazil.

The high cost of adopting the methods is still a relevant barrier, especially in developing countries (total cost = US$ 685.68 ± 28.59, per method), and the values described for monitoring protozoa in WTP residues are substantially more expensive than other routine tests required, such as solids and coliforms.

Table 4 Quotes updated for July 1, 2018, through the General Market Price Index at FGV

Kit enough for 50 samples	Costs (US$)	Unit cost (per sample)	Mean	Standard deviation
Merifluor®	1763.93	35.28	38.16	2.51
	1965.52	39.31		
	1994.08	39.88		
Dynabeads®	5396.62	107.93	114.09	8.71
	6012.34	120.25		

Table 5 Total cost of each method or sample on July 1, 2018, considering the most expensive reagents

Kits and cost	Unit cost per sample (US$)[a]	Unit cost per method (US$)[b]
Merifluor®	76.31 ± 5.02	343.41 ± 22.57
Dynabeads®	114.09 ± 8.71	342.27 ± 26.13
Total cost	190.40 ± 8.10	685.68 ± 28.59

[a] Merifluor®: two units were applied per sample; Dynabeads®: one unit was used per sample

[b] Merifluor®: nine units were applied per method; Dynabeads®: three units were used per method (all the tests were performed in triplicate)

Conclusions

The protocol using CCF with a reduced centrifugation and IMS yielded the highest mean recoveries in the matrix tested, and statistically significant differences were obtained when evaluating *Giardia* spp. In this context, this protocol may be feasible to detect pathogenic protozoa in WTP residues. The other two methods, the first using ICN 7× cleaning solution at 1.0% and the second considering acidification of the sample, both followed by the IMS step, also presented mean recoveries within the stipulated standard. However, the coefficient of variation was high. The aforementioned methods and others should be studied in order to make it possible to detect protozoa in settled residue.

ICN 7× cleaning solution at 1.0% allowed better visualization of the microscope slides, and this method did not involve changes in the pH of the sample. Therefore, it is estimated that testing viability and animal infectivity is more successful when this method is applied. Methods that involve acidification procedures could interfere in the viability; however, they are efficient in matrices with high turbidity.

In the evaluated methods, the second acid dissociation was essential to increase the recoveries of cysts and oocysts in the matrix tested. The efficiency of the methods depends on the matrix. Therefore, more research is needed to evaluate the relevance of the methods in matrices originating from natural sources (i.e., treating water from rivers or lakes).

Detecting protozoa in complex matrices requires high costs, which limit surveillance and control programs in developing countries; therefore, further research is needed to make the parasite detection in complex matrices possible, such as WTP residues.

Compliance with ethical standards

References

American Public Health Association – APHA, American Water Works Association – AWWA & Water Environment Federation – WEF. (2012). *Standard methods for the examination of water and wastewater.* Washington, DC: American Public Health Association.

Andreoli, F. C. & Sabogal-Paz, L. P. (2017). Coagulation, flocculation, dissolved air flotation and filtration in the removal of *Giardia* spp. and *Cryptosporidium* spp. from water supply. *Environmental Technology.* https://doi.org/10.1080/09593330.2017.1400113.

Baldursson, S., & Karanis, P. (2011). Waterborne transmission of protozoan parasites: Review of worldwide outbreaks—an update 2004–2010. *Water Research, 45*(20), 6603–6614.

Campbell, A. T., Robertson, L. J., Smith, H. V., & Girdwood, R. W. A. (1994). Viability of *Cryptosporidium parvum* oocysts concentrated by calcium carbonate flocculation. *Journal of Applied Bacteriology, 76*(6), 638–639.

Chang, C. Y., Huang, C., Pan, J. R., & Wu, B. J. (2007). Modification of immunomagnetic separation procedures for analysis of *Cryptosporidium* at spiked oocysts and turbid sample conditions. *Journal of Environmental Engineering Management, 17*(5), 333–338.

De Oliveira, C. M. B. (2012). Determinação de protocolo para detecção de cistos de *Giardia* spp. e ovos de helmintos, em solos (Determination of a methodology for detection of *Giardia* spp. cysts and helminth eggs, from soil)*,* Master's thesis, Campinas, Brazil, Universidade Estadual de Campinas (University of Campinas).

Efstratiou, A., Ongerth, J. E., & Karanis, P. (2017a). Waterborne transmission of protozoan parasites: Review of worldwide outbreaks—an update 2011–2016. *Water Research, 114*, 14–22.

Efstratiou, A., Ongerth, J., & Karanis, P. (2017b). Evolution of monitoring for *Giardia* and *Cryptosporidium* in water. *Water Research, 123*, 96–112.

Feng, Y., Zhao, X., Chen, J., Jin, W., Zhou, X., Li, N., Wang, L., & Xiao, L. (2011). Occurrence, source, and human infection potential of *Cryptosporidium* and *Giardia* spp. in source and tap water in Shanghai, China. *Applied and Environmental Microbiology, 77*(11), 3609–3616.

Franco, R. M. B., Hachich, E. M., Sato, M. I. Z. S., Naveira, R. M. L., Silva, E. D. C., Campos, M. M. D. C., Neto, R. C., Cerqueira, D. A., Branco, N., & Leal, D. A. G. (2012). Performance evaluation of different methodologies for detection of *Cryptosporidium spp.* and *Giardia* spp. in water for human consumption to meet the demands of the environmental health surveillance in Brazil. *Epidemiologia e Serviços de Saúde (Epidemiology and Health Services), 21*(2), 233–242.

Hachich, E. M., Sato, M. I. Z., Galvani, A. T., Menegon, J. R. N., & Mucci, J. L. N. (2004). *Giardia* and *Cryptosporidium* in source waters of Sao Paulo state, Brazil. *Water Science and Technology, 50*(1), 239–245.

IBGE Instituto Brasileiro de Geografia e Estatística (Brazilian Institute of Geography and Statistics). (2010). *Pesquisa Nacional de Saneamento Básico (National Survey of Basic Sanitation), Rio de Janeiro, Brazil.* https://biblioteca.ibge.gov.br/visualizacao/livros/liv45351.pdf. Accessed 1 July 2018.

Karanis, P., & Kimura, A. (2002). Evaluation of three flocculation methods for the purification of *Cryptosporidium parvum* oocysts from water samples. *Letters in Applied Microbiology, 34*(6), 444–449.

Karanis, P., Schoenen, D., & Seitz, H. M. (1996). *Giardia* and *Cryptosporidium* in backwash water from rapid sand filters used for drinking water production. *Zentralblatt für Bakteriologie, 284*(1), 107–114.

Karanis, P., Shoenen, D., & Seitz, H. M. (1998). Distribution and removal of *Giardia* and *Cryptosporidium* in water supplies in Germany. *Water Science Technology, 37*(2), 9–18.

Keegan, A., Daminato, D., Saint, C. P., & Monis, P. T. (2008). Effect of water treatment processes on *Cryptosporidium* infectivity. *Water Research, 42*(6), 1805–1811.

Koh, W., Clode, P. L., Monis, P., & Thompson, R. A. (2013). Multiplication of the waterborne pathogen *Cryptosporidium parvum* in an aquatic biofilm system. *Parasites & Vectors, 6*(1), 270.

Maciel, P. M. F., & Sabogal-Paz, L. P. (2016). Removal of *Giardia* spp. and *Cryptosporidium* spp. from water supply with high turbidity: Analytical challenges and perspectives. *Journal of Water and Health, 14*(3), 369–378.

Ochiai, Y., Takada, C., & Hosaka, M. (2005). Detection and discrimination of *Cryptosporidium parvum* and *C. hominis* in water samples by immunomagnetic separation-PCR. *Applied and Environmental Microbiology, 71*(2), 898–903.

Olson, M. E., Goh, J., Phillips, M., Guselle, N., & McAllister, T. A. (1999). *Giardia* cyst and *Cryptosporidium* oocyst survival in water, soil, and cattle feces. *Journal of Environmental Quality, 28*(6), 1991–1996.

Rosado-García, F. M., Guerrero-Flórez, M., Karanis, G., Hinojosa, M. D. C., & Karanis, P. (2017). Water-borne protozoa parasites: The Latin American perspective. *International Journal of Hygiene and Environmental Health, 220*, 783–798.

Sato, M. I. Z., Galvani, A. T., Padula, J. A., Nardocci, A. C., de Souza Lauretto, M., Razzolini, M. T. P., & Hachich, E. M. (2013). Assessing the infection risk of *Giardia* and *Cryptosporidium* in public drinking water delivered by surface water systems in Sao Paulo State, Brazil. *Science of the Total Environment, 442*, 389–396.

Sidhu, J. P., & Toze, S. G. (2009). Human pathogens and their indicators in biosolids: A literature review. *Environment International, 35*(1), 187–201.

Steinbaum, L., Kwong, L. H., Ercumen, A., Negash, M. S., Lovely, A. J., Njenga, S. M., Boehm, A. B., Pickering, A. J., & Nelson, K. L. (2017). Detecting and enumerating soil-transmitted helminth eggs in soil: New method development and results from field testing in Kenya and Bangladesh. *PLoS Neglected Tropical Diseases, 11*(4), 1–15.

USEPA. (2012). *Method 1623.1 Cryptosporidium and Giardia in Water by Filtration/IMS/FA*. U.S. Environmental Protection Agency, Washington, DC, USA. Retrieved from: https://nepis.epa.gov/Exe/ZyPURL.cgi?Dockey=P100J7G4.TXT.

Vesey, G., Slade, J. S., Byrne, M., Shepherd, K., & Fricker, C. R. (1993). A new method for the concentration of *Cryptosporidium* oocysts from water. *Journal of Applied Microbiology, 75*(1), 82–86.

Walker, M. J., Montemagno, C. D., & Jenkins, M. B. (1998). Source water assessment and nonpoint sources of acutely toxic contaminants: A review of research related to survival and transport of *Cryptosporidium parvum*. *Water Resources Research, 34*(12), 3383–3392.