Removal of *Giardia* spp. and *Cryptosporidium* spp. from water supply with high turbidity: analytical challenges and perspectives

P. M. F. Maciel and L. P. Sabogal-Paz

ABSTRACT

Giardia and *Cryptosporidium* species are a serious problem if present in water supplies. The removal of these protozoans and the adaptation of existing protocols are essential for supplying drinking water to developing countries. Considering this, the aim of this study is to evaluate, on a bench level, the removal of *Giardia* spp. cysts and of *Cryptosporidium* spp. oocysts from water with high turbidity, using polyaluminium chloride as a coagulant. Filtration using mixed cellulose ester membranes, followed, or not, by purification through immunomagnetic separation (IMS) was used for detecting protozoans. By evaluating the adopted protocol, without using IMS, retrievals of 80% of cysts and 5% of oocysts were obtained, whereas by using IMS, recoveries of 31.5% of cysts and 5.75% of oocysts were reached. When analyzing the coagulant performance, a dosage of 65 mg L^{-1} showed contamination from protozoans in all the samples of filtered water. A dosage of 25 mg L^{-1} presented protozoans in 50% of the filtered water samples. The results showed an improved performance for the 25 mg L^{-1} dosage; therefore, the control of coagulation and adaptation of detection protocols must be evaluated according to the features of raw water and availability of local resources.

Key words | *Cryptosporidium* spp., drinking water, *Giardia* spp., polyaluminium chloride

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INTRODUCTION

In spite of significant improvements in water treatment seen in recent years, there are still reports, including in developed countries, of disease transmission related to the water supply for human consumption (Braeye *et al.* 2014; Puleston *et al.* 2014; Rochelle & Di Giovanni 2014; Widerström *et al.* 2014). Therefore, international agencies have turned their attention to the study and control of emerging microorganisms in light of relevant world events, mainly concerning various outbreaks of cryptosporidiosis and giardiasis (Mac Kenzie *et al.* 1994; Karanis *et al.* 2007; Baldursson & Karanis 2011).

Within this context, assessing the microbiological quality of the water supply requires removing target organisms. These organisms need to be realistic, measurable, based on scientific data, and relevant to local conditions (World Health Organization 2011). Therefore, the *Giardia* and *Cryptosporidium* protozoans are essential for analyzing (using criteria) the drinking water supply. These protozoans appear in the environment in their form of resistance (cysts and oocysts, respectively). In order to complete their life cycle, these organisms need a host. They can cause infection in human beings, where the main symptoms are outbreaks of diarrhea. Furthermore, these organisms show high levels of persistence in aquatic environments (Olson *et al.* 1999). Usually, the presence of these protozoans in water supplies is associated with inadequate domestic sewage disposal and agricultural activities in the drainage basin.

Difficulties in removing cysts and oocysts at water treatment plants (WTPs) are associated, among other factors, with physical characteristics. *Giardia* cysts have dimensions that range from 8 to 18 μm in length and from 5 to 15 μm in width, whereas the diameter of *Cryptosporidium* oocysts

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ranges between 4 and 6 μm (United States Environmental Protection Agency – USEPA 2012). Given these sizes, they are potentially removable through the WTP filters; however, the ability that oocysts have of compressibility (Li *et al.* 1995) can facilitate their passage through the filtering medium, reaching, therefore, the treated water. In addition to the reduced size, the encysted forms are considerably resistant to inactivation by chlorine, a commonly used disinfectant (Korich *et al.* 1990). Thus, maximizing the removal of *Giardia* and *Cryptosporidium* at WTPs is an essential issue (Brown & Emelko 2009). Studies, such as the one carried out by Emelko (2003), demonstrate that coagulation failure could significantly jeopardize the removal of protozoans during the filtration process. However, there are still gaps in the scientific knowledge, such as the variables in the coagulation identified to successfully remove these parasites.

The complexity of the threat of the *Giardia* and *Cryptosporidium* species also involves difficulties in detection. Researchers worldwide have developed protocols for the evaluation of protozoa in environmental samples (Vesey *et al.* 1993; Karanis *et al.* 1998; Franco *et al.* 2001; Karanis & Kimura 2002); nevertheless, a standard procedure is required to provide credibility to results. Method 1623.1 (United States Environmental Protection Agency – USEPA 2012) is validated for detecting protozoans in drinking water and includes sampling, concentration, purification, and detection stages using fluorescence microscopy. The possibility of modifying Method 1623.1 is already foreseen, provided that the analytical quality control of the adopted protocol reaches pre-established criteria. The changes will be accepted when the performance is improved and the costs and sample processing time are reduced (United States Environmental Protection Agency – USEPA 2012).

The reagents, consumption material, and equipment used in Method 1623.1 represent high costs and technical analytical complexity. This fact limits the evaluation of *Giardia* and *Cryptosporidium* in drinking water supplied by Latin American water utilities, even when the law requires or enforces this monitoring. In Brazil, adaptations of Method 1623.1 are being carried out, such as the possibility of concentrating samples using mixed cellulose ester membranes as suggested by Franco *et al.* (2001). This alternative can lead to a reduction in costs, while at the same time complying with international standards. The protocol is already being used in various studies aimed at analyzing the occurrence of *Giardia* cysts and of *Cryptosporidium* oocysts in drinking water and effluents (Franco & Cantusio Neto 2002; Neto *et al.* 2006, 2010; Nishi *et al.* 2009; Cordi *et al.* 2012). However, this protocol has not yet been tested in matrices with high turbidity.

Another possible adaptation of Method 1623.1 may be the use of methods of concentration based on flocculation procedures, according to Vesey *et al.* (1993) and Karanis & Kimura (2002). Both protocols showed high recovery for specific water samples with a low number of oocysts. Nevertheless, such methods may alter the viability of cysts and oocysts, with possible changes in the results of infectivity assays.

The high cost of Method 1623.1 is not restricted only to the concentration phase. Purifying the samples requires immunomagnetic separation (IMS), which can capture cysts and oocysts from a volume of concentrated sample up to 0.5 mL. The volume purified in this procedure can be easily analyzed on microscope well slides. The possibility of eliminating the IMS, complying with international standards, could bring economic benefits to Latin American water utilities, because in Brazil the average cost of the Dynabeads® kit, used on the procedure, is US$5,000.00; one kit is sufficient for only 50 samples (quote obtained in June, 2015).

Regarding the IMS costs, sucrose-flotation methods (Karanis *et al.* 1998) can be attractive to assess protozoa in water samples. Ramirez & Sreevatsan (2006) attained high analytical sensitivity, detecting as few as ten oocysts in water samples. Nonetheless, Santos *et al.* (2004) obtained low recoveries in sewage samples derived from *Giardia* cyst and *Cryptosporidium* oocyst losses during the sucrose purification phase. Research on the performance of sucrose-flotation methods has been developed in Latin America (Quintero-Betancourt & Ledesma 2000; Bonatti *et al.* 2007); however, more studies that include water matrix characteristics and availability of local resources must be performed.

The application of a simplified protocol, which complies with the restrictions of Method 1623.1 in Latin America, will make it possible to estimate the dynamics of these parasites in water, and therefore, measures can be taken to

improve the efficiency of the treatment phases aiming to provide good quality water to the population.

Along these lines, this study assessed the performance of the method by Franco *et al.* (2001), followed, or not, by IMS, aiming to analyze the possibility of reducing the complexity and costs involved in the protocol for detecting *Giardia* spp. cysts and *Cryptosporidium parvum* oocysts. The study also considered the removal of these parasites in Jar-test assays using polyaluminium chloride (PACl) for treating water with high turbidity and the presence of protozoans.

MATERIALS AND METHODS

The study was carried out in three steps. In Step 1, Jar-test assays were carried out to make coagulation diagrams in order to select the optimal points (coagulant dosage vs. pH coagulation, with and without pH correction). Then, the parameters concerning the treatment (rapid-mix, slow-mix, and sedimentation velocity) were optimized. The PACl had an alkalinity of 38.7, Al_2O_3 content of 17.74%, and a pH value of around 3. For the filtration, laboratory filters were used, filled with sand grains (size range from 0.30 to 0.59 mm), which were operated at the filtration rate of 100 $m^3\ m^{-2}\ d^{-1}$. The efficiency of the treatment was evaluated by the filtered water's turbidity. The water from the study, without protozoans, was prepared by adding kaolinite to a water well, at a proportion of 0.1 g L^{-1}, necessary for obtaining a turbidity close to 125 NTU. This study aimed at assessing the performance of the protocol adopted for *Giardia* cysts and *Cryptosporidium* oocysts; therefore, a synthetic water sample was prepared for the elimination of possible interferences in real water samples. Obviously, each laboratory protocol must be adapted to the characteristics of the matrix under study. The physical and chemical analyses carried out followed the procedures described in American Public Health Association – APHA, American Water Works Association – AWWA & Water Environment Federation – WEF (2012).

In Step 2, the analytical quality control method by Franco *et al.* (2001) was performed with adaptations associated with the volume of the sample being processed. Therefore, a known number of cysts and oocysts were inoculated in 1 L of the water from the study. EasySeed® and ColorSeed® were used with the same aim, because the water from the study lacked protozoans. The inoculation was carried out according to the manufacturer's instructions. The water from the study with protozoans was processed according to the method by Franco *et al.* (2001), with and without IMS.

Although methods of concentration, such as calcium carbonate flocculation (Vesey *et al.* 1993) and ferric sulfate flocculation (Karanis & Kimura 2002), could be tested in this study, the protocol of Franco *et al.* (2001) was assessed because it is easier and faster in comparison with flocculation procedures, and the viability of cysts and oocysts can be preserved for future infectivity assays.

The well slide was prepared using the kit from Merifluor® and then scanned using fluorescence microscopy. The number of cysts and oocysts found were compared to the number that was inoculated. Therefore, from the data obtained, the mean recovery percentage and the variation coefficient were estimated and compared to the standard of Method 1623.1.

In Step 3, the performance of the treatment was evaluated (coagulation, flocculation, sedimentation, and filtration), simulated in the Jar-test to remove cysts and oocysts, using the results obtained during Step 1 and Step 2. During this step, the water from the study was inoculated with approximately 500 cysts L^{-1} of *Giardia* spp. and 500 oocysts L^{-1} of *Cryptosporidium parvum* in each Jar-test jar; each jar was previously rinsed using Tween 80 (0.1%). The cysts were purified at the Protozoology Laboratory at the State University of Campinas (Campinas/SP, Brazil) and the oocysts were acquired from Waterborne (USA). The estimate of the number of cysts and oocysts was carried out by evaluating 5 μL of the homogenized concentrated solutions, in triplicate, using the Merifluor kit. The mean value of the readings enabled the quantification of the target organisms present in a determined volume of strain to be inoculated. The aggregation of oocysts, which made it difficult to count, was eliminated by diluting the strain in Tween 20 (0.01%) with subsequent homogenization using a test tube (20 times). Afterwards, the mixture was centrifuged at 1,500 × *g* for 10 min, disposing the supernatant with a subsequent introduction of PBS until the level of 1 mL.

When applying the method by Franco *et al.* (2001), a vacuum pump was used to filter the samples in mixed

cellulose ester membranes (diameter of 47 mm and nominal porosity of 3 μm). Filtration was maintained with a leakage ranging from 1 to 4 L min^{-1} and a pressure of 525 mm Hg. Before being in contact with protozoans, all equipment and glassware were rinsed using Tween® 80 (0.1%). Filtration was carried out until dripping started, always aiming to avoid exceeding the pressure of 600 mm Hg and, at that point in time, the membrane was changed if necessary. After that, the membranes were carefully removed using metal tweezers and then transferred to a tilted Petri dish, from where the retained material was scraped and washed. Scraping was done using a soft plastic strap in parallel movements followed by washing it using 1.0 mL of Tween 80 (0.01%). The yielding liquid was homogenized using a 3.0 mL Pasteur test tube and then transferred to a centrifuge tube. The procedure was repeated twice, keeping the membrane turned at 90 ° from the previous scrapings. The centrifuge tube was filled with Tween 80 up to 50 mL and then centrifuged at $1{,}500 \times g$ for 15 min. After the first centrifugation, the pellet was formed, and then the supernatant was vacuumed leaving 10 mL of sediment in the tube. After this, the liquid was homogenized in vortex and filled up with ultrapure water to submit it to another centrifugation for the same time and at the same rotation rate.

The sample assessed (without IMS) considering disposing of the supernatant after the second centrifugation, leaving a final sediment of 1,000 μL (1.0 mL). The sediment was carefully homogenized before removing the aliquots for disposal on glass well slides. During the procedure, 50 μL of the sediment was divided into five glass wells to analyze the sediment residue, the water from the study and the sediment water. Regarding the filtered water, 75 μL was placed in a single glass well.

The analyzed sample (with IMS) included disposing of the supernatant after the second centrifugation, leaving a sediment of at least 5,000 μL (5.0 mL), depending on the estimated volume of the pellet. The guidelines established in Method 1623.1 were followed when the volume of the pellet was higher than 500 μL. The IMS was processed to capture cysts and oocysts according to the Dynabeads kit manufacturer's instructions by two acid dissociations, aiming to separate the target microorganisms from the magnetic microspheres, and then the slide was prepared using the Merifluor kit. During the procedure, regardless of the sample, the volume transferred to the slide was 100 μL (50 μL at each well considering the two acid dissociations).

The slide was read at a magnification of 200× for identification, and was confirmed at a magnification of up to 800×. Morphological confirmatory aspects were observed by 4′,6-diamidino-2-phenylindole (DAPI) staining and differential interference contrast (DIC) microscopy, at a magnification of 400×. The criteria of Method 1623.1 were followed for characterization of the target organisms. After identification and enumeration, Equation (1) was applied.

$$E = \frac{NxS}{VxA} \times FC \quad (1)$$

where E is the estimate of the number of protozoans (cysts L^{-1} or oocysts L^{-1}); N is the number of cysts or oocysts observed in the well(s) of the immunofluorescence slide; S is the volume of final sediment (μL); V is the total volume of the analyzed sample (L); A is the volume of the aliquot transferred to the well of the slide (μL); and FC is the correction factor only for samples of sediment residue (total volume/concentrated volume).

The performances of the protocol by Franco *et al.* (2001) and of the simulation treatment in the Jar-test (with and without IMS) were weighed according to the analysis of variance (ANOVA test).

RESULTS AND DISCUSSION

Table 1 shows the optimal conditions of the studied water treatment, which was able to generate filtered water with turbidity below 0.5 NTU.

The results of the assays for analytical quality control of the method by Franco *et al.* (2001) are shown in Table 2. The recovery (with and without IMS) did not comply with the requirements of Method 1623.1 for *Cryptosporidium parvum* oocysts.

The loss of cysts and oocysts could be related to the scraping and centrifugation phases. The method tested considered centrifugation at $1{,}500 \times g$ for 15 min; however, Clancy *et al.* (2000) pointed out that values of $2{,}170 \times g$ for 10 min could improve the recovery. Nevertheless, Method

Table 1 | Parameters obtained from the Jar-test assays

Parameter	Without pH adjustment	With pH adjustment
Dosage of PACl (mg L^{-1})	25	65
pH coagulation	6.8	7.2
Mean velocity gradient for rapid mixing (s^{-1})	900 (400 rpm)	1,000 (450 rpm)
Rapid mixing time (s)	15	15
Mean velocity gradient for flocculation (s^{-1})	30 (40 rpm)	20 (30 rpm)
Flocculation time (min)	20	20
Sedimentation velocity (cm min^{-1})	1.5	1.5
Filtration rate (m^3 m^{-2} d^{-1})	100	100

Characteristics of the water from the study: total alkalinity = 36 mg $CaCO_3$ L^{-1}; total aluminium = 0.91 mg L^{-1}; total coliforms = 6 NMP 100 mL^{-1}; *Escherichia coli* = absent; electrical conductivity = 53.7 μS cm^{-1}; hardness = 14 mg $CaCO_3$ L^{-1}; total iron = 0.037 mg L^{-1}; total manganese = 0.008 mg L^{-1}; nitrate = 0.05 mg L^{-1}; nitrite = 0.005 mg L^{-1}; and turbidity = 125 NTU.

Characteristics of the filtered water: total alkalinity = 20 mg $CaCO_3$ L^{-1}; total aluminium < 0.001 mg L^{-1}; total coliforms = absent; *Escherichia coli* = absent; electrical conductivity = 70.8 μS cm^{-1}; hardness = 14 mg $CaCO_3$ L^{-1}; total iron < 0.005 mg L^{-1}; total manganese < 0.003 mg L^{-1}; nitrate < 0.01 mg L^{-1}; nitrite < 0.001 mg L^{-1}; and turbidity < 0.5 TNU.

Table 2 | Results of the analytical quality assays of the method by Franco *et al.* (2001) (with and without IMS) for water from the study

	Recovery mean percentage (number of assays = 4)		Variation coefficient (number of assays = 4)	
Method	*Giardia* spp.	*Cryptosporidium parvum*	*Giardia* spp.	*Cryptosporidium parvum*
Without IMS	80.0%	5.0%	20.4%	200.0%
With IMS	31.5%	5.7%	24.0%	55.7%

1623.1 recommends values in the range from 1,500 × *g* to 2,000 × *g*, with reservations concerning the highest rotations in samples with sediments, as there is the possibility of damaging the target organisms. Another possibility is the difficulty of viewing oocysts through the microscope, since the fluorescent dye can fade when using the Merifluor kit. The same problem was reported by Clancy *et al.* (2000) when assessing seven commercial brands. Intermediate assays for quality control of the method were carried out following guidelines by United States Environmental Protection Agency – USEPA (2012) and the results obtained were similar to those shown in Table 2.

The assays (without IMS) resulted in high recovery efficiencies of *Giardia* spp. cysts (Table 2). The procedure considered the reading of 50 μL of the sample, divided into five wells, from 1,000 μL of sediment. Thus, the organism observed corresponded to 20 organisms of the concentrated sample. This high multiplication factor should be evaluated carefully based on a possible mistake of the estimate. Another aspect that should be considered is the quality of the image viewed through the microscope. The sample presented dense sediment for the 10 μL taken to the well of the slide. This fact created a barrier that made it difficult to view cysts and oocysts and also made the analysis impossible by DIC microscopy and DAPI fluorescence. Based on this, the analysis of the samples that were not submitted to IMS remained restricted to the fluorescein isothiocyanate (FITC) microscopy. This factor represents a limitation of the method without IMS, since algae or other microorganisms could be counted as protozoans if presenting fluorescence, size, and pattern of compatible membranes.

The assays (with IMS) showed a better view of the protozoans through the microscope. Considering this, morphological aspects could be confirmed by eliminating the sample impurities (color by DAPI and visualization by DIC). IMS is advantageous because the sample can be thoroughly analyzed, and therefore the multiplication factor is eliminated. Nevertheless, the procedure entails introducing various steps for the detection protocol, and therefore, when choosing it, the characteristics of the sample, the time of analysis, and its economic viability should be considered. Obviously, the knowledge of the

Table 3 | Removal of *Giardia* spp. cysts and *C.parvum* oocysts using Jar-test assays

		Removal in Log_{10}	
Method	Protozoan	25 mg L^{-1} of PACl (number of assays = 4)	65 mg L^{-1} of PACl (number of assays = 3)
Without IMS	*Giardia* spp.	2.26 ± 0.14	2.31 ± 0.26
With IMS	*Giardia* spp.	3.10 ± 0.41	2.93 ± 0.06
Without IMS	*C. parvum*	1.31 ± 0.35	1.71 ± 0.38
With IMS	*C. parvum*	2.13 ± 0.44	2.19 ± 0.10

Table 4 | Some coagulation variables and characteristics of water from the study and filtered water concerning protozoans

					***Giardia* spp. (cysts.L^{-1})**				***Cryptosporidium parvum* (oocysts.L^{-1})**			
	Coagulation variables		**Turbidity (NTU)**		**With IMS**		**Without IMS**		**With IMS**		**Without IMS**	
Dosage	**pH**	**Zeta potential (mV)[1]**	**WS**	**FW**	**WS**	**FW**	**WS**	**FW**	**WS**	**FW**	**WS**	**FW**
25 mg L^{-1} of PACl (number of assays = 4)	6.70	8.68	130	0.19	576	<1	695	ND	12	ND	17	ND
	6.88	4.74	125	0.25	512	<1	532	ND	14	ND	81	ND
	6.77	8.29	125	0.30	180	ND	517	ND	111	ND	64	ND
	6.70	6.87	132	0.25	79	ND	317	ND	62	<1	100	ND
Mean	6.76	7.15	128	0.25	337		515		50		65	
Standard deviation	0.08	1.78	3.5	0.05	244		155		47		35	
65 mg L^{-1} of PACl (number of assays = 3)	7.07	−13.60	125	0.32	164	<1	333	ND	32	<1	50	ND
	7.40	−16.60	126	0.25	416	<1	450	<2	37	<1	200	<2
	7.40	−14.10	134	1.0	272	<1	1067	ND	252	<1	250	ND
Average	7.29	−14.77	128	0.52	284		617		107		167	
Standard deviation	0.19	1.61	4.9	0.41	126		394		126		104	

[1]Zeta potential of water from the study = −21.8 mV.
ND = non-detected; WS = water from the study; FW = filtered water.

matrix characteristics is important for the evaluation of the best protocols to be selected. In complex matrices, sucrose-flotation methods may be an option for the purification of the samples, however, such an assumption must be tested.

The removal of protozoans, by Jar-test assays, remained in the range of 2 Log_{10}, according to Table 3, which is compatible with Haas *et al.* (2001). Assavasilavasukul *et al.* (2008) reported removals ranging from 0.73 to 5.2 Log_{10} for *Cryptosporidium* spp. and from 1.0 to 5.4 Log_{10} for *Giardia* spp. These results were obtained by treating water through coagulation, flocculation, sedimentation, and filtration. Changes in the concentration of protozoans in the effluent, in the turbidity of the raw water and in the sampling methods contributed to the differences in the observed removals. By analyzing the data obtained, which are presented in Table 3, no statistical difference was observed between the two conditions of coagulation studied by the ANOVA test, based on a reliability level of 95%.

Table 4 shows some variables involved in the coagulation and characteristics concerning protozoans for the water from the study and for the filtered water. The results indicated that the method (without IMS) had a low sensitivity to detect parasites in the filtered water. However, the method (with IMS) for

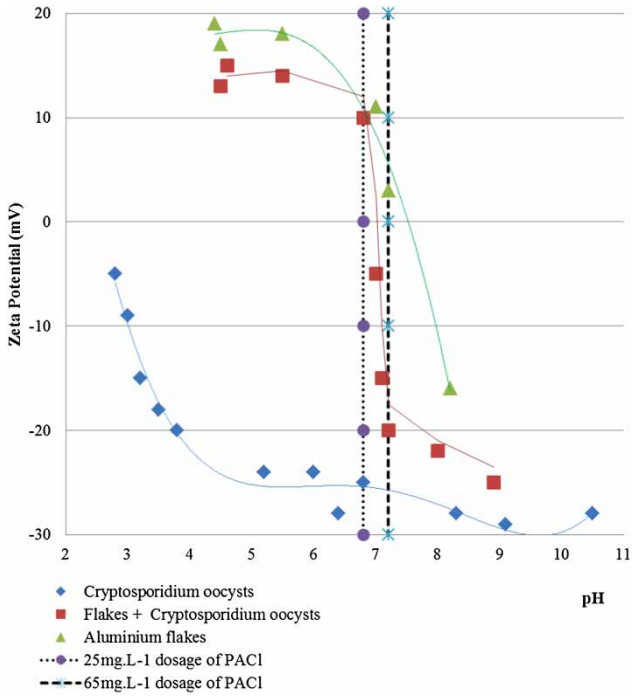

Figure 1 | Effect of pH on the zeta potential of the flakes, oocysts, and oocysts/flakes conjugate (Bustamante *et al.* 2001). The vertical lines represent the pH coagulation for the 25 mg L^{-1} and 65 mg L^{-1} dosages analyzed in this study.

the filtered water, resulting in the dosage of 25 mg L^{-1} of PACl, showed 50% of the samples as positive for cysts and oocysts, whereas for the 65 mg L^{-1} dosage, 100% of the samples presented protozoans.

By assessing Table 4, it can be observed that the pH coagulation is lower for the dosage of 25 mg L^{-1} of PACl. According to Hsu *et al.* (2001), Hsu & Huang (2002), and Tufenkji *et al.* (2006), an increase is expected in the removal of *Cryptosporidium* oocysts by filtering water at a low pH level, since in this condition, it is believed that the zeta potential of oocysts will be less electronegative, therefore favoring their retention. Following this assumption, it is thought that the addition of alkalizing, necessary for the 65 mg L^{-1} of PACl dosage, could have been harmful to the removal of protozoans in the filtering medium. Fernandes *et al.* (2010) assessed the direct filtration technology on a pilot scale, and also observed an increase in the removal of *Cryptosporidium* oocysts in a pH value around 5. Bustamante *et al.* (2001) measured the zeta potential of flakes, oocysts, and the oocysts/flakes conjugate with different pH values, when aluminum sulphate was applied (6.8 mg Al L^{-1}). They observed that the zeta potential was positive for the pH in the range between 4 and 6.8, with a severe decline in the value as the pH was increased (Figure 1).

According to Table 4, dosages of 25 mg L^{-1} and 65 mg L^{-1} of PACl presented a zeta potential of 7.15 ± 1.78 mV at pH values of 6.76 ± 0.08 and of -14.77 ± 1.61 at a pH of 7.29 ± 0.19, respectively. The results warn us that, in conditions close to neutral pH, the coagulation zeta potential is similar to that of the cysts/oocysts/flake conjugate found by Bustamante *et al.* (2001), as depicted in Figure 1. Apparently the reversal of loads, at the point of coagulation, could be the answer to the best performance shown by the 25 mg L^{-1} of PACl dosage, since this could have favored

Table 5 | Percentage distribution of the recovery of cysts and oocysts between the two acid dissociations of IMS

	Samples							
	Raw water		**Settled water**		**Filtered water**		**Sedimented residue**	
	Dissociations (results for *Giardia spp.* cysts)							
Dosage	**1st**	**2nd**	**1st**	**2nd**	**1st**	**2nd**	**1st**	**2nd**
25 mg L^{-1} of PACl (number of assays = 4)	36%	64%	0%	100%	0%	100%	24%	76%
	19%	81%	89%	11%	100%	0%	90%	10%
	45%	55%	0%	0%	0%	0%	56%	44%
	43%	57%	0%	0%	0%	0%	6%	94%
65 mg L^{-1} of PACl (number of assays = 3)	12%	88%	62%	38%	0%	100%	29%	71%
	58%	42%	50%	50%	100%	0%	69%	31%
	15%	85%	0%	100%	0%	100%	53%	47%
Mean	32%	68%	40%	60%	40%	60%	47%	53%
Standard deviation	17%	17%	39%	39%	55%	55%	29%	29%
	Dissociations (results for oocysts of *Cryptosporidium parvum*)							
	1st	**2nd**	**1st**	**2nd**	**1st**	**2nd**	**1st**	**2nd**
25 mg L^{-1} of PACl (number of assays = 4)	62%	38%	0%	0%	0%	0%	0%	100%
	53%	47%	0%	100%	0%	0%	100%	0%
	86%	14%	0%	0%	0%	0%	0%	0%
	67%	33%	0%	0%	100%	0%	17%	83%
65 mg L^{-1} of PACl (number of assays = 3)	59%	41%	100%	0%	100%	0%	0%	100%
	43%	57%	0%	0%	0%	100%	0%	0%
	46%	54%	0%	0%	100%	0%	0%	100%
Mean	59%	41%	50%	50%	75%	25%	23%	77%
Standard deviation	14%	14%	71%	71%	50%	50%	43%	43%

1st = first acid dissociation; 2nd = second acid dissociation.

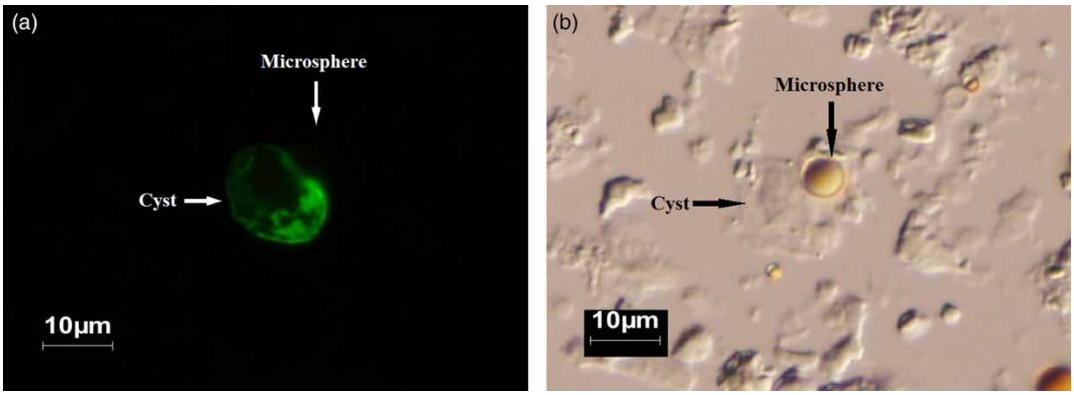

Figure 2 | *Giardia* spp. cyst adhering to the microsphere, at an increase of 400×, viewed in FITC (a) and DIC (b).

the cysts/oocysts/flake conjugate's structure and its retention in the filtrating medium. It is highlighted that the measurement of the zeta potential of the cysts/oocysts' surface is not practical, in contrast to the solution's zeta potential measurement, as was carried out in this study.

In the study, the relevance of the two consecutive acid dissociations, for the method using IMS, was clear (Table 5). The results showed that the second dissociation, when compared to the first one, presented a higher recovery of *Giardia* spp. cysts according to the ANOVA test, at a significance level of 95%. The same behavior was not observed for *Cryptosporidium parvum* oocysts. In spite of this, and regardless of the prevalence of the first or second dissociation, it is essential that this procedure be carried out in two steps.

The data in Table 5 suggest a strong interaction between the cysts and the Dynabeads anti-Giardia microspheres, a phenomenon that can be viewed through the microscope by observing cysts still adhering to microspheres after the acid dissociation (Figure 2). This event was not detected for *Cryptosporidium parvum* oocysts.

The cost of reagents per sample, considering only the Merifluor and Dynabeads kits, was estimated at US$198 and US$190 for assays with and without IMS, respectively (quote in June 2015). Therefore, the use of IMS increased the costs by approximately 4%.

To sum up, the decision regarding using the method of Franco *et al.* (2001) (with and without IMS), should be based on the presence of *Cryptosporidium parvum* oocysts in the raw water; the quality of the water sample; the volume of the sample being processed; the time needed for laboratory analysis; and the costs of the reagents, equipment, and qualified personnel.

CONCLUSIONS

PACl, when used to treat water with high turbidity, enabled us to generate filtered water with turbidity lower than 0.5 NTU. However, *Giardia* spp. cysts and *Cryptosporidium parvum* oocysts were detected in the filtered water. Thus, turbidity is not a variable recommended to control protozoans in drinking water.

The protocol by Franco *et al.* (2001) (with and without IMS), met the standard of Method 1623.1 only for *Giardia* spp. cysts, and the results showed the importance of the second acid dissociation to assess this parasite. Obviously, other simplified methodologies need to be tested, aiming to detect *Cryptosporidium parvum* oocysts in environmental samples in Latin America.

The removal of protozoans, tested by the Jar-test, remained around 2 Log_{10}, and apparently there is better performance for the dosage of 25 mg L^{-1} of PACl, with positive zeta potential, when treating water that has high turbidity and the presence of protozoans.

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