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Research Paper

Direct centrifugation for detecting *Giardia* spp. cysts in filter backwash water

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ABSTRACT

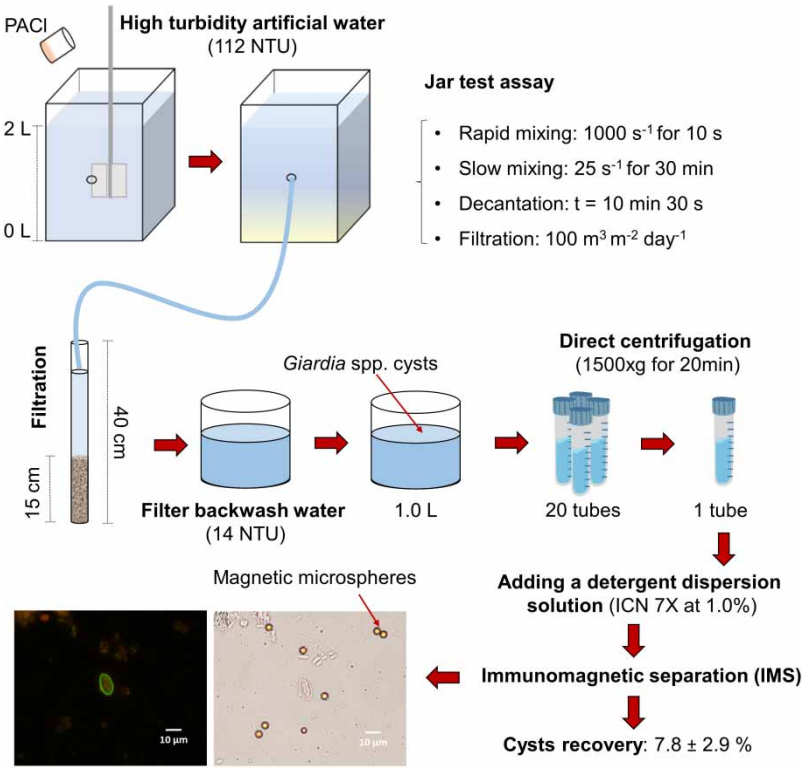
Pathogenic protozoa endanger human health and challenge water treatment, especially during outbreaks in developing countries. For instance, *Giardia* spp. cysts can recirculate in the filter backwash water (FBW), reinforcing the relevance of their detection protocol in negligible matrices. This study aimed to detect *Giardia* spp. cysts in the FBW by direct centrifugation (DC) and immunomagnetic separation (IMS) with the addition of the detergent dispersion solution ICN 7X at 1.0%. To do this, cyst suspensions were inoculated into FBW samples (14 NTU), which was generated in bench-scale drinking-water treatment with high-turbidity study water (112 NTU). Furthermore, the DC+ICN 7X method was compared with the calcium carbonate flocculation. For instance, the DC+ICN 7X method provided cleaner microscope slides and minor damage to the cyst walls. The commercial suspension of *Giardia lamblia* had an adequate recovery rate (19.5%). However, the recovery rate of the EasySeed® suspension was 7.8%, which was below the required range by Method 1623.1 (above 8%). High costs and low efficiencies challenge several methods for detecting *Giardia* spp. cysts. Therefore, future studies should develop and improve detection protocols, especially for complex matrices. Detecting cysts in water treatment residues is crucial for addressing current sanitation issues in developing countries.

Key words: calcium carbonate flocculation, immunomagnetic separation, propidium iodide, protozoa, waterborne disease, water treatment residues

HIGHLIGHTS

- Adding the detergent dispersion solution ICN 7X improved the direct centrifugation (DC) method for concentrating cysts.
- After the immunomagnetic separation, the DC+ICN 7X protocol presented clean reading wells and minor damage to the cyst walls.
- Cyst recoveries were 19.5 and 7.8% for the commercial *Giardia lamblia* and EasySeed® suspensions, respectively.
- Detection protocols for *Giardia* spp. cysts showed low efficiency and high costs.

GRAPHICAL ABSTRACT



INTRODUCTION

Waterborne diseases are of emerging concern, and protozoa have been particularly associated with outbreaks worldwide (Karanis *et al.* 2007; Efstratiou *et al.* 2017b). For instance, *Giardia* spp. cysts are infective forms associated with giardiasis outbreaks (Karanis *et al.* 2007; Baldursson & Karanis 2011). Cyst dimensions range from 7 to 14 μm, and they have low infectious doses in humans, from 1 to 10 organisms (Ortega & Adam 1997). Furthermore, they might present high environmental resistance as their survival has been registered in water for up to 2 months at 8 °C (Cacciò *et al.* 2003).

Waterborne diseases concern developing countries, especially as they may be neglected and associated with inadequate sanitation infrastructure (Coelho *et al.* 2017; Sammarro Silva & Sabogal-Paz 2020). In this scenario, water treatment plants (WTPs) are fundamental in decreasing the drinking-water supply's microbiological risks. Although most cysts can be removed in the filtration steps (Heller *et al.* 2004), further research is still needed to assess the microbial risks of WTP residues, including water treatment sludge (WTS) and filter backwash water (FBW). Moreover, recirculating FBW could reinsert cysts into the treatment system, especially as they resist chlorination, a common disinfection technique (Karanis *et al.* 1996; Freitas *et al.* 2010).

Methods for the detection of *Giardia* spp. cysts can vary significantly among different samples (Franco *et al.* 2012), and most approaches have challenges concentrating and detecting cysts in high-turbidity water. Concentration methods are classified into three categories: filtration (e.g., membranes, microfibers, ultrafiltration, and nanofiltration), flocculation–sedimentation (e.g., calcium carbonate, aluminum sulfate, ferric sulfate, and formaldehyde with ethyl acetate or ether), and centrifugation (e.g., batch and continuous flow) (Efstratiou *et al.* 2017a). Calcium carbonate flocculation (CCF) stands out among concentration methods (Feng *et al.* 2011). This protocol has already been used for high-turbidity water, although pH changes interfere with the viability of the cysts (Giglio & Sabogal-Paz 2018).

The direct centrifugation (DC) with the addition of the detergent dispersion solution ICN 7X (i.e., a neutral and phosphate-free detergent, nontoxic for sensitive organisms) at 1.0% (DC+ICN 7X) concentrates a large volume sample into a pellet (Boni de Oliveira 2012). This protocol was recently applied with the purification by immunomagnetic separation (IMS) for concentrating cysts in high-turbidity water (Giglio & Sabogal-Paz 2018) and WTS (Silva & Sabogal-Paz 2020; Ogura

& Sabogal-Paz 2021a). In addition, another study recovered *Giardia* spp. cysts in the FBW from a bench-scale flotation treatment (Silva & Sabogal-Paz 2020). However, to the best of our knowledge, the literature lacks a protocol for detecting *Giardia* spp. cysts in the FBW from a high-turbidity water treatment. In this scenario, monitoring cysts in WTP is still a challenge for negligible matrices, especially for FBW that can be recirculated without proper disinfection.

Therefore, this research aimed to assess and validate the DC+ICN 7X+IMS method for the detection of *Giardia* spp. cyst in the FBW. The samples were generated in a bench-scale drinking-water treatment, and the experiments were performed with a commercial *Giardia* sp. suspension and the standard EasySeed® (*Giardia lamblia*).

METHODS

This research consisted of two experimental stages: FBW generation by jar test assays and the performance of the DC+ICN 7X+IMS protocol for cyst concentration and detection. In this study, synthetic samples were prepared to minimize interferences from natural freshwater variations (e.g., pH changes, metals, organic matter, and other microorganisms) that might influence experimental conditions (Ogura & Sabogal-Paz 2021b). Physical, chemical, and microbiological parameters were analyzed based on APHA (2012).

FBW generation

The first step consisted of preparing high-turbidity study water (112 NTU) with uncontaminated groundwater and kaolinite (0.16 g L^{-1}, Sigma-Aldrich®/Fluka 60609). Then, the bench-scale drinking-water treatability assays were performed in jar tests (2 L) with automatic fast-mixing (1,000 s^{-1} for 10 s) and slow-mixing (25 s^{-1} for 30 min). The polyaluminium chloride (PACl) was selected as the coagulant (specific weight of 1.362 g L^{-1} and 16.68% content of Al_2O_3). Three PACl dosages were tested (24, 25, and 26 mg L^{-1}, corresponding to 2.11, 2.20, and 2.29 mg Al^{3+} L^{-1}, respectively) based on previous studies (Giglio & Sabogal-Paz 2018). The chosen decantation rate was 1.5 cm min^{-1} (i.e., approximately 10 min 30 s). Then, the clarified water was directed to the filtration stage.

Second, the filtration step was performed in attached laboratory filters (ALF). The ALF consisted of acrylic tubes with a 19 mm internal diameter, a 40 cm height, and previously washed and dried sand (grain size between 0.30 and 0.59 mm, and effective size of 0.42 mm). The clarified water from each jar was directed to one ALF, and the adopted filtration rate was 100 m^3 m^{-2} day^{-1}. Finally, each sand filter media was washed with 300 mL of distilled water to generate the FBW. Multiple treatability assays were performed to obtain a compound FBW sample.

Cyst counting and inoculation

All material was washed with a 0.1% Tween 80 solution to avoid cyst adhesion to solid surfaces. The commercial suspension of *Giardia* sp. was purified at the Laboratory of Protozoology at the State University of Campinas, Brazil. The suspension was homogenized in a vortex (2 min) before each use. The counting of the average number of cysts followed the recommendations from the Merifluor® kit (Meridian Bioscience, Inc.) with DAPI (4′,6-diamidino-2-phenylindole) solution (Sigma-Aldrich®, F6057). The procedures were done with an aliquot of 5 μL of the suspension in triplicate. In addition, potentially viable cysts were identified by the propidium iodide (PI) incorporation (Sigma-Aldrich®, P4170). The volume of PI solution corresponded to the added suspension (5 μL), and cysts were classified as stained (PI-positive) or not-stained (PI-negative) (Ogura & Sabogal-Paz 2021a). The counting was performed in a microscope, considering DAPI, fluorescence (fluorescein-5-isothiocyanate, FITC), PI, and differential interference contrast (DIC) images.

The volume of suspensions was inoculated directly into the FBW samples (1 L), considering the estimated number of cysts and the target concentration. This procedure was chosen to avoid losses throughout the treatment process (e.g., flocculation and filtration). The samples were then homogenized with a magnetic stirrer for 20 min. First, a preliminary analytical quality assay (n=3) was performed with the commercial suspension of *Giardia* sp. (with a target concentration of $\pm$2,000 cysts L^{-1}). Second, the analytical quality control (n=4) validated the chosen method with the EasySeed® (BTF Bio – Australia) suspension (with a pre-determined number of 100 $\pm$ 1.9 *G. lamblia* cysts).

Concentration protocols

The DC+ICN 7X method was adapted from Boni de Oliveira (2012). First, the contaminated FBW sample (1 L) was divided into 20 tubes (Falcon®, 50 mL). Then, the centrifugation step was conducted (at 1,500×g for 20 min), and the remaining pellets (0.5 mL) were transferred to a single tube. In addition, all tubes were washed with 0.5 mL of Tween 80 (0.1%), and this content was transferred to a single tube. Next, another centrifugation was carried out, and the supernatant was removed until

the remaining 5 mL mark. This pellet was transferred to a flat-sided tube (FST) with 3.0 mL of 1.0% ICN detergent dispersion solution 7X (ICN Pharmaceuticals Inc.®). After homogenization in a rotatory mixer (20 rpm for 1.0 h), the FST content was transferred to a centrifuge tube (Falcon®, 50 mL). The last centrifugation (1,500×*g* for 20 min) concentrated the sample into a 5 mL pellet.

The CCF protocol was adapted from Giglio & Sabogal-Paz (2018). These procedures also aimed to concentrate 1.0 L of FBW into a 5 mL sample. First, 10 mL of calcium chloride ($CaCl_2$, 1 M) and 10 mL of sodium bicarbonate ($NaHCO_3$, 1 M) were added to the FBW. After the samples were agitated for 10 min, 1.5 mL of sodium hydroxide (NaOH, 5 M) increased the pH to 10. Then, the samples rested overnight (at room temperature, covered with a watch glass). On the next day, the supernatant was removed until the 100 mL mark, and 20 mL of 10% sulfamic acid (H_3NSO_3) was added. This sample was divided into three centrifuge tubes (Falcon®, 50 mL). The beaker was washed with 30 mL of Tween 80 (0.1%), and this content was distributed to the tubes. After centrifugation (20 min at 1,500×*g*), the supernatant was discarded from each tube. The remaining pellets (1.0 mL) were transferred to a single tube. The pH was corrected to neutrality (7.0), and 4.2 mL of PBSS (phosphate-buffered saline solution – Sigma-Aldrich®) was added. The volume was concentrated by centrifugation (10 min at 1,500×*g*) into a 5 mL remaining pellet.

Both methods of concentration were followed by the IMS purification protocol (USEPA 2012) according to the Dynabeads™ GC-Combo (IDEXX®) procedures and two acid dissociations. The United States Environmental Protection Agency Method 1623.1 for drinking water was used as guidance to validate the recovery efficiency (USEPA 2012). Finally, statistical analyses were performed on Statistica®. First, the Shapiro–Wilk verified the normality and homogeneity, respectively. Then, statistical differences were evaluated by Student's *t*-test and the analysis of variance (ANOVA), with 95% confidence (p-value<0.05).

RESULTS AND DISCUSSION

Water treatment and FBW generation

The groundwater used in this study had total alkalinity of 15.3 mg $CaCO_3$ L^{-1}, conductivity of 33.2 μS cm^{-1}, turbidity of 0.2 NTU, an apparent color of 0.0 HU, and a pH of 6.78. After adding 0.16 g L^{-1} of kaolinite, the study water had total alkalinity of 8.8 mg $CaCO_3$ L^{-1}, conductivity of 53.2 μS cm^{-1}, turbidity of 112 NTU, apparent color of 114 HU, true color of 3.3 HU, and a final pH of 7.15.

The filtered water's apparent color and turbidity were endpoints to evaluate the treatment efficiency (Table 1). The dosage of 2.11 mg Al^{3+} L^{-1} had lower average apparent color (1.5 HU) for filtered water in 10 min but with a higher standard deviation (±1.4 HU). Therefore, the dosage of 25 mg PACl L^{-1} (2.20 mg Al^{3+} L^{-1}) was applied to achieve low-turbidity filtered water (0.18±0.1 NTU, after 20 min); then, this dosage was chosen for the following research steps. The reference study used to estimate this dosage (Giglio & Sabogal-Paz 2018) under similar treatability conditions and study water (125 and 130 NTU, respectively) also considered 2.20 mg Al^{3+} L^{-1} as the optimal PACl dosage for obtaining 0.25 and 0.18 NTU filtered water, respectively.

Table 1 | Apparent color (HU) and turbidity (NTU) for the samples collected (n=3) in the treatability test in jar tests for three PACl dosages

	PACl dosage (mg Al^{3+} L^{-1})					
	2.11		2.20		2.29	
Samples	Apparent color (HU)	Turbidity (NTU)	Apparent color (HU)	Turbidity (NTU)	Apparent color (HU)	Turbidity (NTU)
Study water	66.7±2.9	118.3±6.1	67.4±1.9	114.3±7.2	66.9±2.4	113.3±11.1
Clarified water	2.6±0.6	1.4±0.4	3.3±0.8	1.4±0.2	2.7±0.2	1.6±0.1
Filtered water (after 10 min)	1.5±1.4	0.3±0.1	1.7±0.3	0.2±0.1	2.1±0.6	0.4±0.2
Filtered water (after 20 min)	2.1±0.7	0.2±0.1	1.8±0.1	0.2±0.1	1.8±0.1	0.3±0.2
FBW	4.2±3.8	4.1±3.0	7.5±2.6	6.2±0.8	9.0±4.5	5.2±2.5

After the water treatment assays, the generated FBW (compound sample) presented turbidity of 14.5 NTU, apparent color of 9.1 HU, true color of 1.2 HU, pH 6.10, conductivity of 52.1 $\mu S\ cm^{-1}$, 113 mg L^{-1} of total solids, 0.94 mg L^{-1} of total organic carbon, and alkalinity of 12.3 mg $CaCO_3\ L^{-1}$, 0.55 mg Al L^{-1}, and 0.374 mg Fe L^{-1}. Further information on drinking-water treatment conditions and FBW characterization was previously presented in Ogura & Sabogal-Paz (2021b).

Approximately 1,900 cysts L^{-1} were inoculated into the FBW samples, considering the cysts counting on the suspension. The preliminary assay (Table 2) showed recovery rates of 14.4 and 10.7% for CCF and DC+ICN 7X, respectively. Thus, the recoveries from our study complied with Method 1623.1 (USEPA 2012), which establishes at least an 8% recovery rate. Furthermore, the DC+ICN 7X recovered more PI-negative cysts (86 cysts, 83.5% of the total) than CCF (43 cysts, 27.4%). This observation may result from CCF method damage to cyst cell walls through pH changes (Giglio & Sabogal-Paz 2018). In these cases, indicator dyes (e.g., PI) can be incorporated into *Giardia* spp. cysts through cell wall disruptions (Campbell *et al.* 1992). Thus, the DC+ICN 7X method was selected as it showed cleaner wells and recovered more PI-negative cysts (Figure 1). Nonetheless, this was a preliminary assay with only one sample for each method.

The DC+ICN 7X+IMS method recovered 19.5 ± 0.6 and 7.8 ± 2.9 cysts for the commercial and EasySeed® suspensions (statistically different as $p<0.05$), respectively (Table 3). Thus, our study's protocol for cyst detection complied with Method 1623.1 (USEPA 2012). However, the commercial suspensions presented more consistent results, with a higher

Table 2 | Preliminary assay with CCF and DC+ICN 7X detection protocols, followed by IMS with two acid dissociations to compare the recovery of *Giardia* spp. cysts artificially inoculated in the FBW

	Number of cysts recovered			Recovery (%)		
Recovery from detection protocols	**First dissociation**	**Second dissociation**	**Total**	**First dissociation**	**Second dissociation**	**Total**
CCF+IMS	83	74	157	7.6	6.8	14.4
DC+ICN 7X+IMS	60	43	103	6.2	4.5	10.7
	Number of PI-negative cysts recovered			**PI-negative cysts from the recovered (%)**		
PI-negative cysts from detection protocols	**First dissociation**	**Second dissociation**	**Total**	**First dissociation**	**Second dissociation**	**Total**
CCF+IMS	15	28	43	18.1	37.8	27.4
DC+ICN 7X+IMS	59	27	86	98.3	62.8	83.5

CCF, calcium carbonate flocculation; DC, direct centrifugation; ICN 7X, ICN detergent dispersion solution 7X; IMS, immunomagnetic separation.

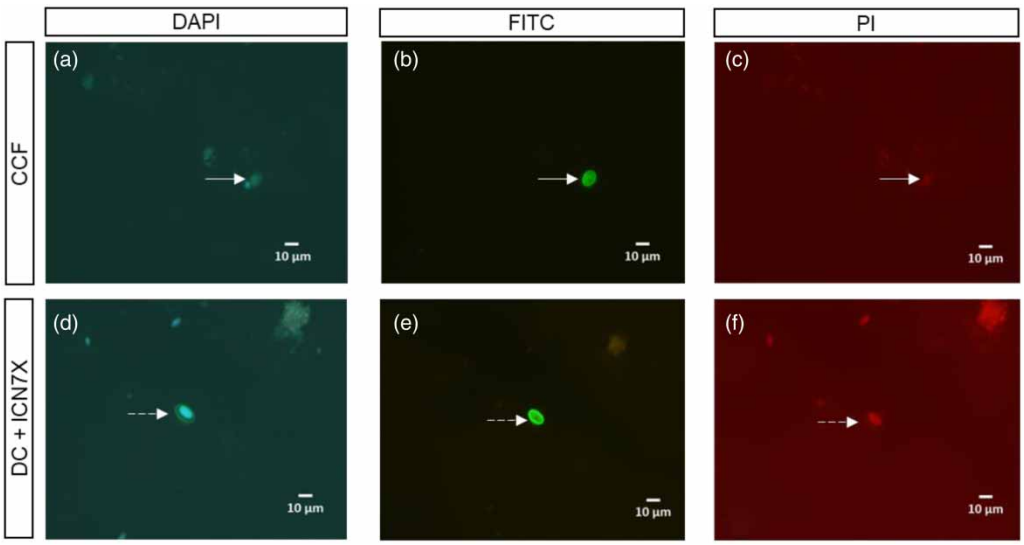


Figure 1 | Microscopy images from FBW for the preliminary assays with the concentration methods CCF (a–c) and DC+ICN 7X (d–f). Non-stained (PI-negative) *G. lamblia* cysts indicated with arrows and stained (PI-positive) cysts indicated with dashed arrows. Images (400×): DAPI (a and d); FITC (b and e); and PI (c and f). CCF, calcium carbonate flocculation; DC, direct centrifugation; ICN 7X, ICN detergent dispersion solution 7X; DAPI, 4′,6-diamidino-2-phenylindole; FITC fluorescein-5-isothiocyanate; PI, propidium iodide.

Table 3 | Average recoveries and coefficient of variation for the analytical quality assays with *Giardia* spp. suspensions detected by the DC+ICN 7X+IMS protocol for the FBW, considering stained (S) (PI-positive) and not-stained (NS) (PI-negative) cysts

	Commercial suspension (inoculated cysts in the FBW: 1,900 cysts L^{-1})							
Samples	First dissociation		Second dissociation		Total number			
Replicates	NS	S	NS	S	NS	S	Total recovery (%)	Non-stained cysts (%)
1	72	159	25	117	97	276	19.6	26.0
2	102	167	11	101	113	268	20.1	29.7
3	65	143	18	132	83	275	18.8	23.2
Average	79.7	156.3	18.0	116.7	97.7	273.0	19.5	26.0
Standard deviation	19.7	12.2	7.0	15.5	15.0	4.4	0.7	3.3
Coefficient of variation	24.7	7.8	38.9	13.3	15.4	1.6	3.4	12.4
	EasySeed® suspension (inoculated cysts in the FBW: 100.0 ± 1.9 cysts L^{-1})							
Samples	First dissociation		Second dissociation		Total number		Total recovery (%)	
Replicates	S		S		S			
1	3		5		8		8.0	
2	2		5		7		7.0	
3	3		1		4		4.0	
4	10		2		12		12.0	
Average	4.5		3.3		7.8		7.8	
Standard deviation	3.2		1.8		2.9		2.9	
Coefficient of variation	71.1		54.9		36.9		36.9	

DC, direct centrifugation; ICN 7X, ICN detergent dispersion solution 7X; IMS, immunomagnetic separation.

recovery rate (19.5%) and a lower coefficient of variation (3.2%) than EasySeed® (7.8 and 36.9%, respectively). In addition, most cysts were recovered in the first dissociation for the commercial suspensions ($p<0.05$), while the number was higher from the second dissociation with EasySeed® ($p<0.05$). Ultimately, Andreoli & Sabogal-Paz (2021) indicated that a third acid dissociation increases the number of recovered cysts up to 31.9% from flotation FBW samples. The present investigation was limited to two acid dissociations in the IMS protocol due to the high costs of analyses.

Other studies performed several protocols for detecting cysts from FBW with turbidity ranging from 7 to 27 NTU (Table 4). For instance, Silva & Sabogal-Paz (2020) applied the DC+ICN 7X+IMS method and achieved a 43.9% average recovery rate of *Giardia* spp. (from a commercial suspension) for FBW (26.7 NTU) from a flotation bench-scale jar test. However, these authors reported a recovery rate of only 3.8% for the EasySeed® suspension, which was lower than our study (7.8% for a 14 NTU FBW). This variability can result from different FBW samples generated from specific water treatment processes (i.e., decantation and flotation). In addition, the ferric sulfate flocculation method was applied for *Giardia muris* in FBW (6.6 NTU) and reached an 1.8% recovery rate (Sammarro Silva & Sabogal-Paz 2021). In another study, these authors applied membrane filtration and obtained a recovery rate of 17.4% of *G. muris* cysts from the same FBW sample, although adding the IMS protocol did not improve the recovery rate of this concentration method (13.0%) (Sammarro Silva & Sabogal-Paz 2020).

Furthermore, for the FBW from swimming pools, Greinert *et al.* (2004) had a 3.3% recovery rate for *Giardia* spp. cysts when combining the CCF and DC protocols. Although Ladeia *et al.* (2018) did not find *Giardia duodenalis* in their FBW samples, cysts were detected in 6.1% of their centrifuged and WTS samples. In addition, high-turbidity water has also been studied (Table 4). For example, Giglio & Sabogal-Paz (2018) achieved an 11.5% cyst recovery rate for this protocol for high-turbidity study water (130 NTU). These authors showed that the CCF and DC+H_2SO_4 methods presented higher average recovery rates (46.1 and 26.0%).

Table 4 | A comparison of *Giardia* spp. analytical quality assay results from the literature regarding detection protocols in water treatment residues (i.e., FBW and WTS) and high-turbidity water

Detection protocol	Matrix	Turbidity (NTU)	Protozoa	Suspension	AR (%)	CV (%)	Reference
DC+ICN 7X+IMS	FBW (Decantation)	14	*Giardia* spp.	Commercial	19.5	3.2	This study
			G. lamblia	EasySeed®	7.8	36.9	
	FBW (Flotation)	27	*Giardia* spp.	Commercial	43.9	25.5	Silva & Sabogal-Paz (2020)
			G. lamblia	EasySeed®	3.8	60.0	
	Floated residue	380	*Giardia* spp.	Commercial	32.5	29.5	
			G. lamblia	EasySeed®	49.0	2.0	
	WTS	3,187	*Giardia* spp.	Commercial	17.0	22.2	Ogura & Sabogal-Paz (2021a)
			G. lamblia	EasySeed®	24.8	32.4	
		538	*G. muris*	Commercial	0.0	>100	Sammarro Silva & Sabogal-Paz (2020)
	High-turbidity water	130	*Giardia* spp.	Commercial	11.5	85.5	Giglio & Sabogal-Paz (2018)
CCF	WTS	538	*G. muris*	Commercial	0.3	100	Sammarro Silva & Sabogal-Paz (2020)
CCF+IMS	FBW	14	*Giardia* spp.	Commercial	14.4	n.d.	This study
	High-turbidity water	130	*Giardia* spp.	Commercial	46.1	5.0	Giglio & Sabogal-Paz (2018)
	WTS	538	*G. muris*	Commercial	0.2	23.6	Sammarro Silva & Sabogal-Paz (2020)
CCF+DC	FBW (from swimming pools)	n.d.	*Giardia* spp.	Commercial	3.3	n.d.	Greinert *et al.* (2004)
DC+H_2SO_4	High-turbidity water	130	*Giardia* spp.	Commercial	26.0	16.3	Giglio & Sabogal-Paz (2018)
$Al_2(SO_4)_3$+IMS	WTS	538	*G. muris*	Commercial	4.2	27.8	Sammarro Silva & Sabogal-Paz (2020)
$Fe_2(SO_4)_3$+IMS	WTS	538	*Giardia* spp.	ColorSeed®	32.2	9.0	
$Fe_2(SO_4)_3$+IMS	FBW	7	*G. muris*	Commercial	1.8	43.7	Sammarro Silva & Sabogal-Paz (2021)
	WTS	538	*G. muris*	Commercial	6.4	31.9	Sammarro Silva & Sabogal-Paz (2020)
Membrane filtration	FBW	7	*G. muris*	Commercial	17.4	39.6	
Membrane filtration+IMS	FBW	7	*G. muris*	Commercial	0.0	61.0	
	FBW	7	*Giardia* spp.	ColorSeed®	13.0	19.6	
Method 1623.1	Drinking water		*Giardia* spp.		8–100	≤39	USEPA (2012)

n.d., not determined; DW, drinking water; AR, average recovery; CV, coefficient of variation; DC, direct centrifugation; IMS, immunomagnetic separation.

Most research has focused on artificial FBW samples under controlled laboratory conditions (Table 4). Accordingly, in the present investigation, synthetic water was used to minimize possible adverse effects and interferences of the matrix on recovery, as the objective was to evaluate the detection method itself. However, the studied protocol should be tested and validated in other matrices; notably, various FBW samples generated from raw freshwater (e.g., water samples with different organic matter content and turbidity). In addition, the DC+ICN 7X+IMS protocol could be applied to actual contaminated FBW samples as the inoculation of protozoa suspensions into artificial samples from the laboratory might not represent the conditions from WTPs. Finally, the variations from commercial and EasySeed® suspensions are still unclear, although different results from our research could be related to the number of inoculated cysts in each protocol. Studies have already claimed that higher initial concentrations of cysts would likely result in better recovery efficiencies (Sammarro Silva & Sabogal-Paz 2020 and references therein). These aspects could be interesting for future studies regarding the gaps in detecting *Giardia* spp. cysts in FBW samples.

The WTS is another residue from WTP that has been studied with several detection protocols (Table 4). For instance, Ogura & Sabogal-Paz (2021a) concentrated *Giardia* spp. cysts from a WTS sample (3,187 NTU) with 17.0 and 24.8% recoveries, respectively, for the commercial and EasySeed® suspensions. Furthermore, these authors applied the DC+ICN 7X+IMS

protocol for detecting cysts after the alkaline treatment with 27 mg CaO/100 mL. As a result, they achieved 2.05 and 2.14 logs of cyst inactivation for 3 and 5 days, respectively. The interferences on the viability of cysts can pose challenges for evaluating the disinfection efficiency (Silva & Sabogal-Paz 2020; Ogura & Sabogal-Paz 2021a). These studies considered PI-negative cysts to indicate potential viability when assessing alkaline and ozone treatments for disinfection of water treatment residues. On the other hand, Silva & Sabogal-Paz (2020) showed 32.5 and 49.0% recoveries for the floated residue (380 NTU) by applying the DC+ICN 7X+IMS protocol. This detection protocol is also viable for other challenging matrices. For example, Boni de Oliveira (2012) obtained 37.6 and 33.2% recovery rates for concentrating soil samples with 500 and 1,000 cysts of *Giardia* spp., respectively. Accordingly, Orlofsky *et al.* (2013) applied centrifugation and IMS for soil samples, recovering up to 89 ± 11% of *G. lamblia* cysts.

Magnetic microspheres were detected in the reading wells even after two acid dissociations (Figure 2(d)). This limitation was also observed by Andreoli & Sabogal-Paz (2021), who detected cysts that were still attached to the magnetic microspheres after three acid dissociations. Furthermore, Fava *et al.* (2021) applied the microfiltration followed by the IMS protocol with 50% fewer beads for water with 40 NTU. As a result, these authors obtained a 56.1% recovery rate for a commercial *G. duodenalis* cyst suspension, while 47.5% for the analytical quality control with ColorSeed®. Thus, 50 µL of the immunomagnetic beads complied with Method 1623.1 for *G. duodenalis* (USEPA 2012), which is an advantage regarding cost reduction.

Cryptosporidium spp. oocysts are another concern for protozoa monitoring and detection in developing countries. In a previous study (Ogura & Sabogal-Paz 2021b), *Cryptosporidium* spp. oocysts were recovered from FBW samples generated under the same treatment conditions. However, oocyst recovery rates (15.4 ± 3.3 and 2.8 ± 0.8%) were lower than the ones obtained for *Giardia* spp. cysts in the present research (19.5 ± 3.2 and 7.8 ± 36.9%), considering commercial and Easyseed® suspensions, respectively. Although Method 1623.1 requires a higher oocyst recovery rate (>32%) than cysts (>8%), this observation was also reported by another evaluation of both protozoa in FBW samples (Silva & Sabogal-Paz 2020).

The costs incurred by cyst detection in WTP residues were challenging in the present research. Four studies performed the cost analysis for the IMS protocol, ranging from US$ 118 to US$ 212 (Giglio & Sabogal-Paz 2018; Ogura & Sabogal-Paz 2021a). In addition, Andreoli & Sabogal-Paz (2021) indicated that a third acid dissociation had a 23.5% increase in costs compared to the second one, which might not be feasible. Therefore, high costs concern and limit protozoa environmental monitoring, especially in developing countries with limited or unevenly distributed financial resources.

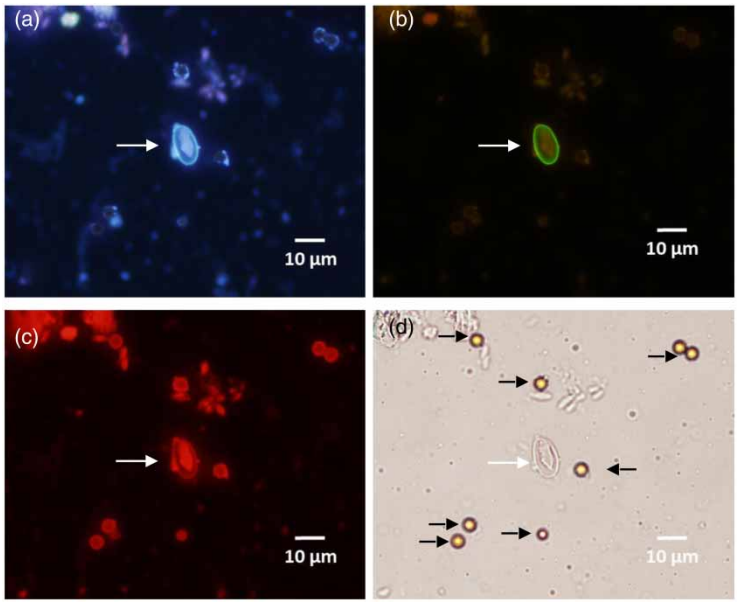


Figure 2 | Stained (PI-positive) *G. lamblia* cyst from the DC+ICN 7X EasySeed® analytical quality control. Parasite indicated with arrows and microspheres with a dashed arrow. Images (400×): (a) DAPI, (b) FITC, (c) PI, and (d) DIC. DC, direct centrifugation; ICN 7X, ICN detergent dispersion solution 7X; DAPI, 4′,6-diamidino-2-phenylindole; FITC, fluorescein-5-isothiocyanate; PI, propidium iodide; DIC, differential interference contrast.

CONCLUSIONS

This study performed the DC+ICN 7X+IMS protocol to detect *Giardia* spp. cysts from an artificially contaminated FBW, which was generated by the bench-scale water treatment. The cyst recovery rate (7.8%, considering the EasySeed® suspension) from the applied protocol was slightly below the required range by Method 1623.1 (8–100%). On the other hand, the recovery rate was superior for the commercial suspension (19.5%), meeting the minimum standards. Nonetheless, other studies from the literature presented recovery efficiencies generally below 50%, highlighting some challenges for *Giardia* spp. cyst detection in water treatment residues. Therefore, the high costs and low efficiency show an evident limitation of this methodology. Moreover, developing feasible detection protocols for *Giardia* spp. cysts can contribute to studies on protozoa outbreaks and giardiasis epidemiology. In addition, future studies should apply this methodology to natural samples (e.g., surface water and effluents from the wastewater treatment).

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CONFLICT OF INTEREST STATEMENT

The authors certify that they have no affiliations with or involvement in any organization or entity with any financial or non-financial interest in the subject matter or materials discussed in this manuscript.

DATA AVAILABILITY STATEMENT

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

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