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), generated in bench-scale drinking-water treatment with a high turbidity study water (112 NTU). Furthermore, the DC + ICN 7X method was compared to 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 (19.5%). However, the recovery rate of the EasySeed® suspension was 7.8%, 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.

Keywords

Calcium Carbonate Flocculation; Immunomagnetic Separation; Propidium Iodide; Protozoa; Water Treatment Residues; Waterborne Disease.

Highlights

- Adding the detergent dispersion solution ICN 7X improved the direct centrifugation (DC) method for concentrating cysts.
- After the immunomagnetic separation (IMS), 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.

Introduction

Waterborne diseases are of emerging concern, and protozoa have been particularly associated with outbreaks worldwide (Efstratiou et al. 2017b; Karanis et al. 2007). For instance, *Giardia* spp. cysts are infective forms associated with giardiasis outbreaks (Baldursson and Karanis 2011; Karanis et al. 2007). Cyst dimensions range from 7 to 14 µm, and they have low infectious doses in humans, from 1 to 10 organisms (Ortega and Adam 1997). Furthermore, they might present high environmental resistance as their survival has been registered in water for up to two 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 and Sabogal-Paz 2020). In this scenario, water treatment plants (WTP) 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 (Freitas et al. 2010; Karanis et al. 1996).

Methods for *Giardia* spp. cysts detection can significantly vary 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 and Sabogal-Paz 2018).

The direct centrifugation with the addition of the detergent dispersion solution ICN 7X (i.e., a neutral and phosphate-free detergent, non-toxic 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 and Sabogal-Paz 2018) and WTS (Ogura and Sabogal-Paz 2021a; Silva and Sabogal-Paz 2020). In addition, another study recovered *Giardia* spp. cysts in the FBW from a bench-scale flotation treatment (Silva and 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 *Giardia* spp. cyst detection 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® (*G. 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 and Sabogal-Paz 2021b). Physical, chemical, and microbiological parameters were analyzed based on APHA (2012).

Filter backwash water 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 (1000 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 and 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 to

generate the FBW. Multiple treatability assays were performed to obtain a compound FBW sample.

Cysts 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 from 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 and 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 ± 2000 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 ± 1.9 *Giardia 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 1500xg for 20min), 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 the single tube. Next, another centrifugation was carried out; and the supernatant was removed until the remaining 5 mL. 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 (1500xg for 20 min) concentrated the sample into a 5 mL pellet.

The CCF protocol was adapted from Giglio and 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 1500xg), 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 1500xg) 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 the 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}, a conductivity of 33.2 μS cm^{-1}, turbidity of 0.2 NTU, an apparent color of 0.0 HU, and pH 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 the final pH was 7.15.

The filtered water's apparent color and turbidity were endpoints to evaluate treatment efficiency (Table 1). The dosage of 2.11 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 and 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 polyaluminium chloride (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
Filter backwash water	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 µS cm^{-1}, 113 mg L^{-1} of total solids, 0.94 mg L^{-1} of total organic carbon, 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 and Sabogal-Paz (2021b).

Approximately 1900 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 8% recovery. 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 and Sabogal-Paz 2018). In these cases, indicator dyes (e.g., propidium iodide - 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

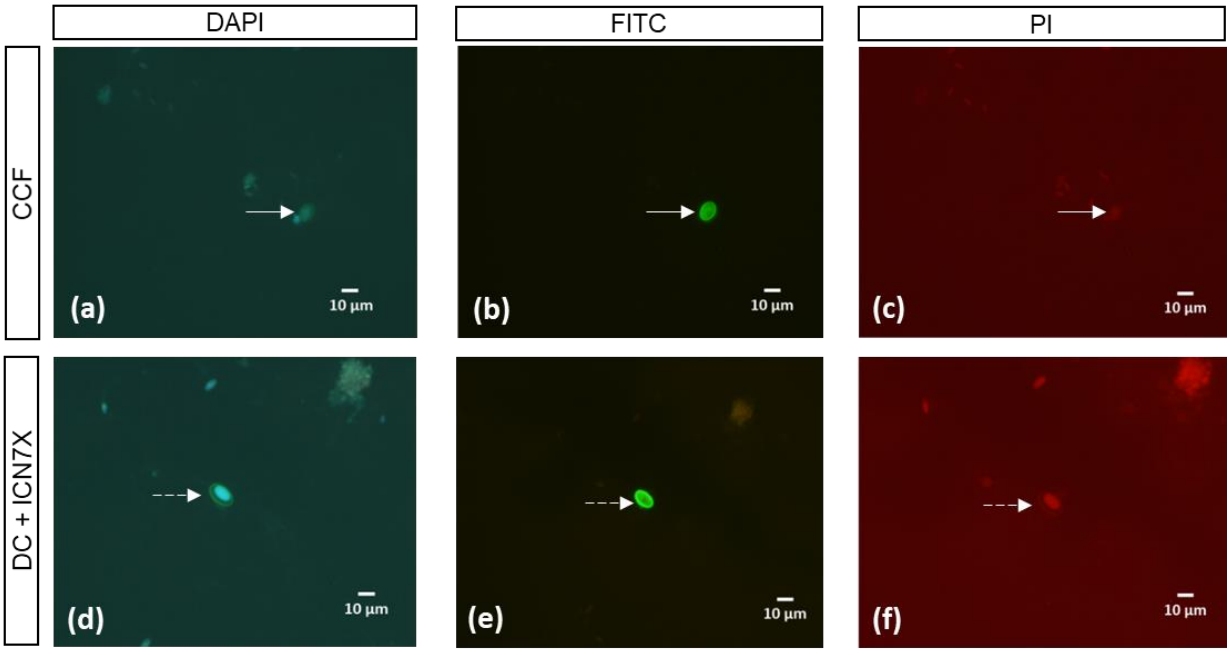
showed cleaner wells and recovered more PI-negative cysts (Figure 1). Nonetheless, this was a preliminary assay with only one sample for each method.

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 filter backwash water.

Recovery from detection protocols	Number of cysts recovered			Recovery (%)		
	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
PI-negative cysts from detection protocols	Number of PI-negative cysts recovered			PI-negative cysts from the recovered (%)		
	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

Notes: 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, b, c) and DC + ICN 7X (d, e, f). Non-stained (PI-negative) *Giardia lamblia* cysts indicated with arrows and stained (PI-positive) cysts indicated with dashed arrows. Images (400X): DAPI (a and d); FITC (b and e); PI (c and f).



Notes: 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.

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 higher recovery (19.5%) and 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 and 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.

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 filter backwash water (FBW), considering stained (S) (PI-positive) and not-stained (NS) (PI-negative) cysts.

	Commercial suspension (Inoculated cysts in the FBW: 1900 cysts L^{-1})							
Samples	First dissociation		Second dissociation		Total number		Total recovery (%)	Non-stained cysts (%)
Replicates	NS	S	NS	S	NS	S		
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

Notes: DC = direct centrifugation; ICN 7X = ICN detergent dispersion solution 7X; IMS = Immunomagnetic separation.

Other studies performed several protocols for detecting cysts from FBW with turbidity ranging from 7 to 27 NTU (Table 4). For instance, Silva and Sabogal-Paz (2020) applied the DC + ICN 7X + IMS method and achieved a 43.9% average recovery 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 of only 3.8% for the EasySeed® suspension, 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 a 1.8% recovery (Sammarro Silva and Sabogal-Paz 2021). In another study, these authors applied membrane filtration and obtained a recovery of 17.4% of *G. muris* cysts from the same FBW sample, although adding the IMS protocol did not improve the recovery of this concentration method (13.0%) (Sammarro Silva and Sabogal-Paz 2020).

Table 4 – A comparison of *Giardia* spp. analytical quality assay results from the literature regarding detection protocols in water treatment residues (i.e., filter backwash water and water treatment sludge) and high turbidity water.

Detection Protocol	Matrix	Turbidity (NTU)	Protozoa	Suspension	AR (%)	CV (%)	Reference
DC + ICN 7X + IMS	Filter backwash water (Decantation)	14	*Giardia* spp.	Commercial	19.5	3.2	This study
			G. lamblia	EasySeed®	7.8	36.9	
	Filter backwash water (Flotation)	27	*Giardia* spp.	Commercial	43.9	25.5	(Silva and 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	
	Water treatment sludge	3187	*Giardia* spp.	Commercial	17.0	22.2	(Ogura and Sabogal-Paz 2021a)
			G. lamblia	EasySeed®	24.8	32.4	
		538	*G. muris*	Commercial	0.0	> 100	(Sammarro Silva and Sabogal-Paz 2020)
	High turbidity water	130	*Giardia* spp.	Commercial	11.5	85.5	(Giglio and Sabogal-Paz 2018)
CCF	Water treatment sludge	538	*G. muris*	Commercial	0.3	100	(Sammarro Silva and Sabogal-Paz 2020)
CCF + IMS	Filter backwash water	14	*Giardia* spp.	Commercial	14.4	n.d.	This study
	High turbidity water	130	*Giardia* spp.	Commercial	46.1	5.0	(Giglio and Sabogal-Paz 2018)
	Water treatment sludge	538	*G. muris*	Commercial	0.2	23.6	(Sammarro Silva and Sabogal-Paz 2020)
CCF + DC	Filter backwash water (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 and Sabogal-Paz 2018)
$Al_2(SO_4)_3$ + IMS	Water treatment sludge	538	*G. muris*	Commercial	4.2	27.8	(Sammarro Silva and Sabogal-Paz 2020)
$Fe_2(SO_4)_3$ + IMS	Water treatment sludge	538	*Giardia* spp.	ColorSeed®	32.2	9.0	
$Fe_2(SO_4)_3$ + IMS	Filter backwash water	7	*G. muris*	Commercial	1.8	43.7	(Sammarro Silva and Sabogal-Paz 2021)
	Water treatment sludge	538	*G. muris*	Commercial	6.4	31.9	(Sammarro Silva and Sabogal-Paz 2020)
Membrane filtration	Filter backwash water	7	*G. muris*	Commercial	17.4	39.6	
Membrane filtration + IMS	Filter backwash water	7	*G. muris*	Commercial	0.0	61.0	
	Filter backwash water	7	*Giardia* spp.	ColorSeed®	13.0	19.6	
Method 1623.1	Drinking water		*Giardia* spp.		8-100	≤ 39	(USEPA 2012)

Notes: n.d. = not determined; DW = drinking water; AR = average recovery; CV = coefficient of variation; DC = direct centrifugation; IMS = Immunomagnetic separation

Furthermore, for the FBW from swimming pools, Greinert et al. (2004) had a 3.3% recovery for *Giardia* spp. cysts when combining the CCF and DC protocols. Although Ladeia et al. (2018) did not find *G. 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 and Sabogal-Paz (2018) achieved 11.5% cyst recovery for this protocol for a high turbidity study water (130 NTU). These authors showed that the CCF and DC + H_2SO_4 methods presented a higher average recovery (46.1 and 26.0%).

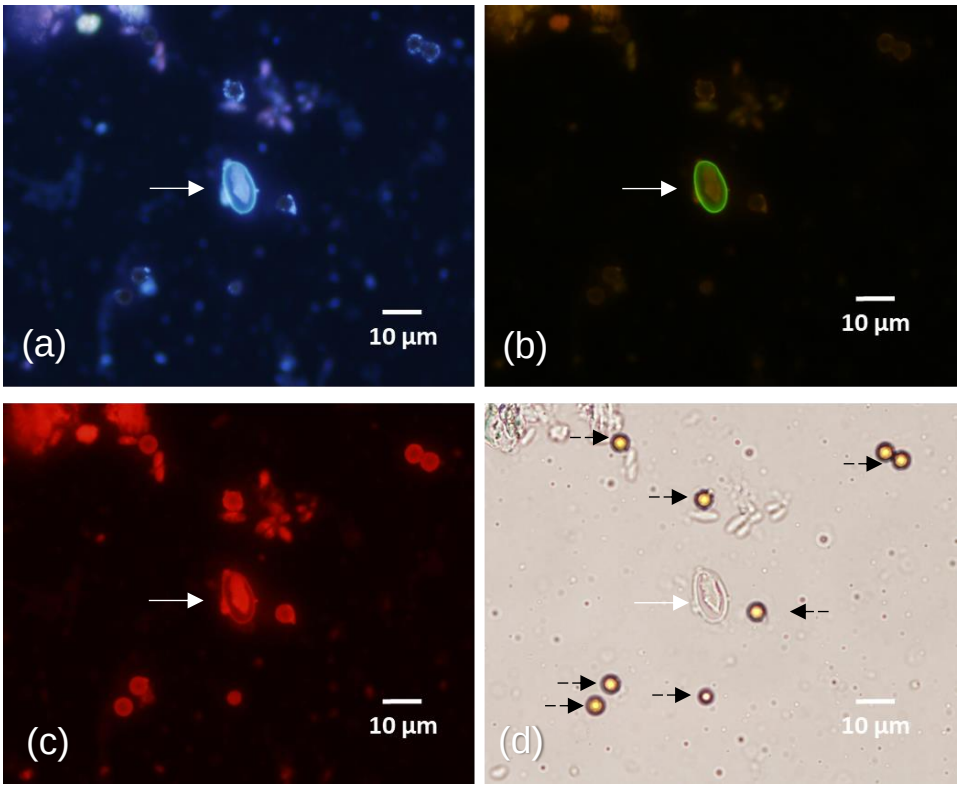
Most research has focused on artificial FBW samples under controlled laboratory conditions (Table 4). Accordingly, in the present investigation, synthetic water was used to minimize possible adverse effects and interferences of the matrix on recovery, as the objective was to evaluate the detection method 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 + ICN7X + 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 and 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 and Sabogal-Paz (2021a) concentrated *Giardia* spp. cysts from a WTS sample (3187 NTU) with 17.0 and 24.8% recoveries, respectively, for a commercial and the EasySeed® suspension. 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 (Ogura and Sabogal-Paz 2021a; Silva and Sabogal-Paz 2020). 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 and 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 1000 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 2d). This limitation was also observed by Andreoli and 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 for a commercial *Giardia 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.

Figure 2 - Stained (PI-positive) *Giardia lamblia* cyst from the DC + ICN 7X EasySeed® analytical quality control. Parasite indicated with arrows and microspheres with a dashed arrow. Images (400X): (a) DAPI; (b) FITC; (c) PI; (d) DIC.



Notes: 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.

Cryptosporidium spp. oocysts are another concern for protozoa monitoring and detection in developing countries. In a previous study (Ogura and Sabogal-Paz 2021b), *Cryptosporidium* spp. oocysts were recovered from FBW samples generated under the same treatment conditions. However, oocyst recovery (15.4 ± 3.3% and 2.8 ± 0.8%) was 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 higher oocyst recovery (> 32%) than cysts (> 8%), this observation was also reported by another evaluation of both protozoa in FBW samples (Silva and 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 and Sabogal-Paz 2018; Ogura and Sabogal-Paz 2021a). In addition, Andreoli and 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.

Conclusions

This study performed the DC + ICN 7X + IMS protocol to detect *Giardia* spp. cysts from an artificially contaminated FBW, generated by bench-scale water treatment. The cyst recovery (7.8%, considering the EasySeed® suspension) from the applied protocol was slightly below the required range by Method 1623.1 (8 to 100%). On the other hand, the recovery 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. cysts 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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Data Availability Statement: The authors confirm that the data supporting the findings of this study are available within the article.

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