Giardia spp. cysts and *Cryptosporidium* spp. oocysts in drinking water treatment residues: comparison of recovery methods for quantity assessment

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Abstract

Water treatment plant (WTP) residues, e.g. sludge and filter backwash water (FBW), may contain pathogenic microorganisms, as *Giardia* spp. and *Cryptosporidium* spp.. However, recovering protozoa from such matrices lacks a formal and precise protocol, which is imperative to improve research in their detection, removal and inactivation. The latter includes a deeper challenge as some recovery methods may compromise viability. This study applied different recovery methods for *G. muris* cysts and *C. parvum* oocysts spiked into settled sludge and FBW obtained from a bench treatment. Procedures in sludge involved direct centrifugation, alkaline and acid flocculation, including purification by immunomagnetic separation (IMS). FBW samples were tested for membrane filtration (MF) and heated Tween® scrapings followed or not by IMS. Propidium iodide (PI) inclusion was used for oocyst viability evaluation prior and after recovery. Results with purified suspensions lead to higher recovery efficiencies (RE) for *C. parvum*, which was assumed to relate to poor *G. muris* fluorescence. Analytical quality assessments were carried out with ColorSeed® for the methods that stood out for each matrix and the results indicated lower RE than when organisms from purified suspensions were recovered. Ferric sulfate flocculation and MF, both followed by IMS reached 32.25 and 11.00% RE for *Giardia* spp. and 19.61 and 2.00% for *Cryptosporidium* spp., respectively. All of the tested methods affected oocyst viability. These results encourage further research to overcome the matrices complexity explained in this paper and increase RE, taking effects in protozoa viability into consideration.

Keywords water treatment sludge; filter backwash water; protozoa; propidium iodide; drinking water

Introduction

Parasitic infections are responsible for mortality and morbidity due to acute diarrhea. These cases occur in a large part of the world's population, affecting particularly low-income people in developing countries, and are therefore called "neglected tropical diseases", among which are those caused by bacteria, helminths and protozoa [1–3].

The genera of protozoa *Giardia* and *Cryptosporidium* include the most commonly encountered enteric parasites, responsible for gastrointestinal diseases causing nutritional imbalances and severe health problems, especially in children and people with immunological deficiencies [4]. The diseases caused by *Giardia* and *Cryptosporidium* are called giardiasis and cryptosporidiosis, respectively.

Recent studies have mentioned giardiasis and cryptosporidiosis cases in North America [5], Europe [6], Asia [7–9], Africa [10,11], and South America [1]. In these researches, such diseases were associated with low family income, vulnerable populations and lack of sanitation infrastructure. This is because effluents and residues may constitute dissemination sources for microorganisms, and contain high concentration of protozoan cysts and oocysts, thus they may contaminate water bodies and affect public supply [12,13]. These parasites cause a vicious cycle of exposure to poverty and insalubrity, as they compromise child development and worker productivity, which requires greater attention from the sanitation and health sectors.

Cysts and oocysts are resistant to conventional disinfection methods, which endorses the need to properly remove them from water [14–16]. In conventional water treatment plants (WTPs), the main removal routes are decantation (or flotation) and filtration. As these are separation processes, they generate waste, i.e. sludge and filter washing water, where impurities are concentrated, as well as microorganisms of similar size [17].

The absence of adequate management of these residues poses a risk to public health, particularly in a scenario in which there is sludge dumping in water bodies and recycling of untreated filter back-wash water (FBW) to the system [17]. Thus, the microbiological characterization of WTPs residues may assess and encourage new policies towards its management.

Protozoan cysts and oocysts detection, however, is challenging because, in addition to the high costs involved, there is an analytical limitation in the recovery methods, which present low reproducibility and require experts to perform the assays [18–20]. Also it is imperative

that the recovery procedure do not compromise inferences in (oo)cyst viability [21], a parameter often used to assess inactivation.

Method 1623.1, established by the United States Environmental Protection Agency [22] stands for cysts and oocysts recovery in water, and its analytical quality requires a minimum recovery of 8% for *Giardia* spp. and 32% for *Cryptosporidium* spp., which are low values themselves and may underestimate the presence of these protozoa in the water under analysis. As turbidity values and solids concentration increase, identification of the target organisms becomes even more difficult [23]. As for residues from WTPs, which have different physical and chemical characteristics when compared to water, there is no defined procedure for detecting cysts and oocysts [20], and that implies even greater subjectivity to the interpretation of protozoan occurrence data in such matrices.

In this scene, this study aimed at comparing recovery methods for *Giardia* and *Cryptosporidium* in WTP sludge and FBW in terms of recovery rates and effect on membrane permeability, therefore assessing viability loss caused by the recovery procedure alongside to its efficiency.

Materials and methods

Matrices preparation

The first step of the methods considered a series of jar-test assays using a surface water source (average turbidity of 30.7±5.2 NTU and apparent color of 76.6±9.9 uH). The applied dosage of polyaluminium chloride (PACl 17.51% Al_2O_3), used as coagulant, was 10 mg L^{-1} under optimal conditions previously tested: rapid mixing under a gradient of 700 s^{-1} for 10 s; slow mixing under 30 s^{-1} for 20 min; and settling velocity of 2.0 cm min^{-1}. After decanting, the jars were drained, in order to allow collecting 100 mL from the bottom of each jar, which were then mixed into one vessel and submitted to gravity densification during a 30 min settling, generating the studied sludge.

Another set of treatability bench tests was carried out in the same condition, but including a filtration step after decanting. Laboratory filters, filled with sand grains (size range from 0.30 to 0.59 mm), were coupled to the jar-test equipment and operated at the filtration rate of 100 m^3 m^{-2} d^{-1} [19]. After filtrating approximately 350 mL of clarified water, the filters were washed, generating the filter backwash water (FBW) for this study. Filter washing was carried out by the insertion of deionized water in ascending direction (using a 50 mL syringe). Then, the entire content of the filter (sand and interstitial water) were poured into a beaker and 100

ml of deionized water was added to help washing the sand. The supernatant of approximately 150 mL for each filter was collected, representing the studied FBW.

Target organisms

Sludge and filter backwash water samples of 100 mL and 1000mL, respectively, were spiked with organisms for the recovery assays (n=4 and a blank sample, in which there was no inoculum). The target organisms were inoculated from aliquots of purified suspensions of live *Giardia muris* cysts and *Cryptosporidium parvum* oocysts (Waterborne, Inc.). The cysts and oocysts were obtained from stool samples of experimentally infected CFW® mice and calves, respectively, by sucrose and Percoll density gradient centrifugation. The estimate number of organisms was given by enumeration (in triplicate) of the inoculated volumes directly into microscopy glass slides, which was performed in parallel to each recovery test.

The methods that led to statistically higher recovery rates (Shapiro-Wilk normality check and t-Student test for 95% confidence interval) were repeated for analytical quality assessment, using organisms from ColorSeed^{TM} (BTF, Ltd.), as recommended by Method 1623.1 (USEPA 2012). ColorSeed^{TM}, which contains a defined number of 100 cysts of *Giardia* spp. and 100 oocysts of *Cryptosporidium* spp.., was inoculated into the samples following the manufacturer's protocol and the assays had the same number of repetitions as the prior (n=4 and blank test).

After spiking, the samples were mildly homogenized with a magnetic stirrer for 30 min. All vessels and materials that were exposed to the protozoa were previously rinsed with Tween® 80 elution solution (0.1% v v^{-1}).

Recovery assays

Different sets of recovery assays were performed for each matrix, considering the feasibility associated to the physical and chemical characteristics of the two WTP residues under analysis (sludge and FBW). A general representation of the selected methods is shown in Figure 1.

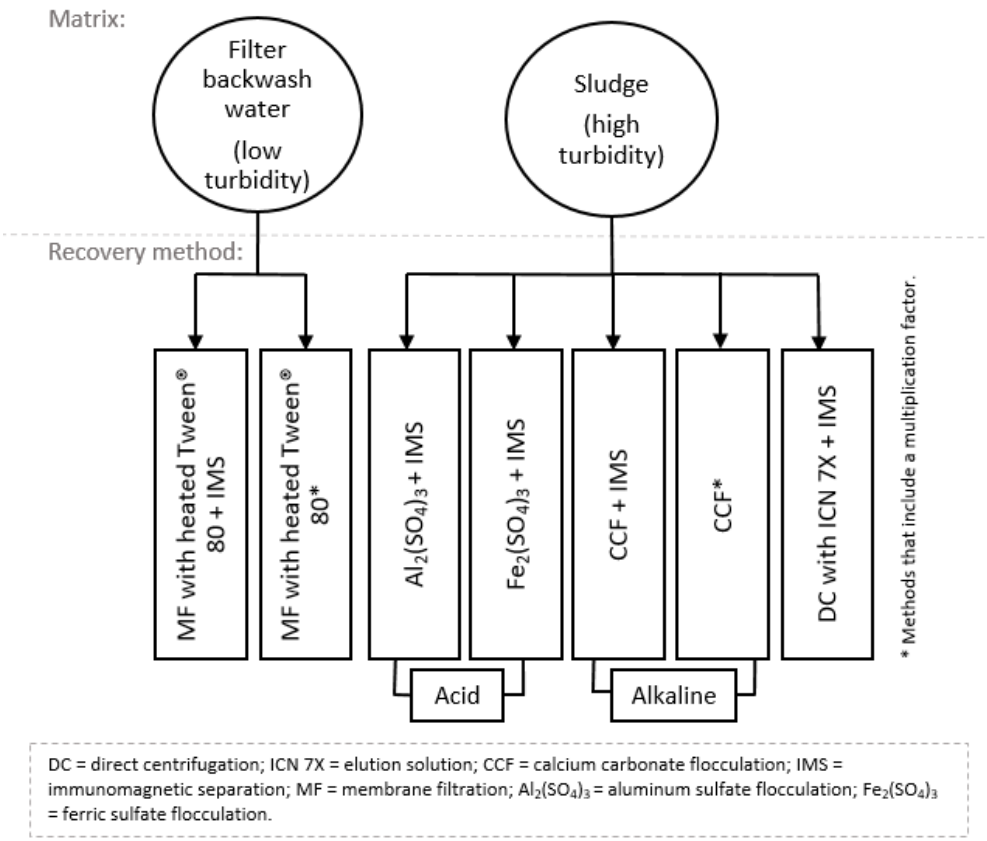

Figure 1 – General diagram of the recovery assays for cysts and oocysts

The spiked protozoa from sludge samples were recovered by different centrifugation and flocculation methods, followed or not by an immunomagnetic purification step (IMS). As for FBW, a filtration method was carried out, also considering the effect of the selective separation afterwards.

Direct centrifugation

Sludge samples were concentrated by direct centrifugation (DC) (1,500 x g; 20 min) with ICN 7x cleaning solution at 1% (v v^{-1}), adapted from Giglio and Sabogal-Paz [20]. In order to do so, the samples were divided into centrifuge tubes (Falcon® tubes of 50 mL capacity) and then submitted to a first centrifugation run. After that, the supernatant of each tube was carefully removed, preserving the pellet at approximately 5 mL. For each repetition, both tubes had their pellets homogenized in vortex, then one of the tubes was emptied and rinsed with 5 mL of ICN 7X, so that its content could be poured into only one Falcon® tube. The remaining sample (approximately 15 mL) was then submitted to a second centrifugation. The supernatant, above the 5 mL mark, was discarded and the remaining pellet was again disaggregated by vortexing and subjected to purification.

Purification was performed by immunomagnetic separation (IMS) (Dynabeads®, Dynal® CG, Combo anti-*Cryptosporidium* and anti-*Giardia*, Applied Biosystems) according to the

manufacturer's recommendations. However, it was taken into account that the matrix derived from a surface water source and contained metals from both the water body and the coagulant used for the treatment. Therefore, an extra step preceded the IMS, in which each sludge sample was homogenized in order to suspend the pellet obtained after the DC and transfer it to a flat side tube (FST) (Dynal^{TM}). Each FST was then coupled to a magnetic concentrator (Dynal MPC^{TM}-1) to perform manual motions willing to adhere any magnetic particles (e.g. precipitates of metal salts) to the MPC and remove them from the sludge sample. The sample volumes were preserved in this step, which was included prior to all of the IMS procedures in this study.

Flocculation methods

Four different flocculation methods were carried out for the sludge concentration in this study. Two of them had an alkaline basis, as the applied flocculant was calcium carbonate followed or not by IMS. The other methods were acid-based and had aluminum sulfate or ferric sulfate as flocculants, both followed by the purification step.

In more detail, an adaptation of the calcium carbonate flocculation (CCF) method [24,25] was carried out for the 100 mL sludge samples. Calcium chloride ($CaCl_2$, 1 mL 1 M) and sodium bicarbonate ($NaHCO_3$, 1 mL 1 M) were added to the samples under stirring. Subsequently, the samples' pH was raised to 10 by sodium hydroxide (NaOH) (the required volume was checked prior to protozoal inoculation). The samples were left to settle for 4 hours and then the supernatant was carefully removed until the 30 mL indication. The concentrate was then stirred for homogenization and, thereafter, sulfamic acid (H_3NSO_3, 2 ml at 10% v v^{-1}) was added under stirring. Each sample was then transferred to a 50 mL Falcon® tube, which also contained the volume of Tween® 80 used to rinse the beaker. The content was centrifuged at 1,500 x g for 20 min. The supernatant was discarded up to 10 mL and the tube was filled with ultrapure water and centrifuged again (1,500 x g, 20 min). The supernatant was carefully removed, preserving 5 mL, which was then vortexed for homogenizing. The centrifugation tube was filled with PBS (phosphate saline solution, pH 7.4) and the sample was submitted to a third centrifugation, from which a 5 mL sediment was preserved. From this point on, the samples were vortexed and homogenized and either aliquots were taken for microscopy or the whole volume was purified by IMS. For the recovery assay that only considered concentration of the samples, Equation 1 lead to the recovery results:

$$R = \left(\frac{\sum_{i=1}^{i=n} P \times \mathrm{F}}{\mathrm{NP}}\right) \times 100\% \quad (1)$$

Where: R = recovery (%); P = number of cysts or oocysts visualized in each microscopy well; NP = average number of cysts or oocysts inoculated in the samples; F = correction factor equal to 10, calculated by the ratio of the volume of the concentrated sample and the aliquot under study. When the CCF + IMS method took place, the recovery efficiency did not require a correction coefficient.

As for acid flocculation, both tested methods included selective separation by IMS. The difference in the two procedures was the chosen flocculant for the concentration step: either ferric sulfate or aluminum sulfate, which have both been described by Karanis and Kimura and Kourenti et al. [26,27].

In this study, flocculation by ferric sulfate was carried out by adding $Fe_2(SO_4)_3$ to the sludge samples in order to obtain a final Fe^{3+} concentration of 5 mg L^{-1}. Thereafter, the pH of the samples was lowered to 6.0 by adding hydrochloric acid (the required volume was checked prior to inoculating protozoa). The sludge samples were kept standing for 4 hours and then the supernatant was carefully removed, leaving approximately 25 mL of sample and its sediment, which were stirred for 10 min for homogenization. Each sample was transferred to a 50 mL Falcon® tube, which also contained the volume of Tween® 80 used to rinse the beaker. The content was centrifuged at 2,000 x g at 4 °C for 10 min and the centrifugation supernatant was removed in order to keep a 1 mL pellet. It was resuspended by vortexing with 1 mL of lysis buffer (solution of 8.4 g of citric acid monohydrate and 17.64 g of sodium citrate diluted in 100 mL of ultrapure water; buffered to pH 4.7). The pellets were left to settle with the buffer for 60 min (vortexed every 15 min). Three ultrapure water washes were performed, to remove possible sample droplets on the centrifuge tube wall, as well as to increase the volume to approximately 5 mL. The sample was centrifuged again (2,000 x g, 10 min, 4 °C) and the supernatant discarded until the 1 mL label. PBS (pH 7.4) was added until the 5 mL label of the centrifuge tube and it was vortexed. Achieving neutral pH, the volume of 5 mL was purified by IMS.

A similar procedure was carried out for aluminum sulfate, in which $Al_2(SO_4)_3$ was added to the sludge samples in order to obtain a final Al^{3+} concentration of 16 mg L^{-1} and the pH was adjusted to 5.4. The same settling times, lysis buffer and centrifugation conditions were applied in comparison to ferric sulfate flocculation. The final 5 mL samples were also purified by IMS.

Filtration methods

Membrane filtration (MF) of 1,000 mL samples of FBW (n=4 + blank test) was performed with cellulose mixed esters membranes (47 mm diameter and 3 μm nominal porosity, Millipore®), adapted from Franco et al. [28]. It required a vaccum pump under a 4 L min^{-1} flow rate and 500 mmHg pressure. The organisms were eluted through scrapping of the membrane using three repetitions of Tween 80 (0.1%, 45 °C) rinsing. The concentration was carried out by centrifugation at 1,500 x g for 15 min of the elution solution content for each sample. This method was considered with and without purification by immunomagnetic separation. When only concentration by MF was carried out, recovery rates were calculated by Equation 1, considering the aliquot volume analyzed from the final pellet. The assay that included IMS considered a 5 mL concentrate that was used for the purification procedure and the recovery efficiency did not consider a multiplication coefficient.

Detection and viability assessment

Protozoan quantification was performed by immunofluorescence assay (IFA) using the Merifluor® kit and the samples were examined under 400x magnification, using an epifluorescence microscope (Olympus® BX51). Morphological confirmatory aspects were observed by 4'6-diamidino-2 phenylindole (DAPI) staining, following according to Method 1623.1 [22] recommendations.

Viability assessment considered as membrane integrity was tested by propidium iodide (PI) exclusion, which has been widely applied in matrices such as treated wastewater [29], agricultural waste [30] and drinking-water [31]. PI counts were performed for both the inoculum (as a reference quantified in parallel to sample spiking) and the recovered samples. Suspension ages were obtained by the difference between the registered date in which the recovery assay was performed in relation to the date of the purification from stool samples. This information was logged as a reference to compare some expected die-off to viability loss. The optical filters used for the organisms detection and analysis are shown in Table 1.

Table 1. Optical filters used for protozoa visualization

Optical filter	Max. excitation wavelength (nm)	Max. emission wavelength (nm)
Fluorescein isothiocyanate (FITC)	490	520
4′,6-diamidino-2-phenylindole (DAPI)	385	420
Propidium iodide (PI) filter	528	617

Thus, slide preparation followed Merifluor® manufacturer's protocol, with extra steps for nuclei labeling with DAPI and viability assessment by PI exclusion. After slide staining with the Merifluor® reagents, two drops of Fluoroshield™ with DAPI (F6057, Sigma-Aldrich®)

were added to each slide well and left for staining for 10 min in the absence of direct light. This was followed by three slide washings using 50 µL of Hank's Balanced Salt Solution (HBSS, H6648, Sigma-Aldrich®). Similarly, PI (P4170, Sigma-Aldrich®) solution (1 mg L^{-1}) was added to each well in the same volume as the sample and left for 5 min in the absence of direct light. Each well was then rinsed with 50 µl of HBSS and 50µL of ultrapure water, in order to avoid crystal formation. Samples that contained ColorSeed® for analytical quality analysis were not stained with PI.

Results and discussion

Table 2 displays the WTP residues characteristics, compared to the raw water. It indicates FBW has a lower ion presence (inferred by conductivity measures as well as aluminum and iron ions), which may be due to the distilled water used for filter washing. The low values for solids reached for this matrix was compatible with membrane filtration, selected for later (oo)cyst recovery assays. As for sludge, the obtained physical and chemical properties endorsed the idea that decantation, in a conventional WTP is one of the main routes for clarification and it must therefore contain a concentrated number of pathogens such as protozoa.

Table 2 – Characteristics of the water treatment plant simulated residue compared to the raw water.

Parameter	Unit	Raw water	FBW	Sludge
Turbidity	NTU	27.2	6.64	538
Apparent color	uH	113.0	8.5	-
Conductivity	µS cm^{-1}	43.96	15.95	48.72
Total alkalinity	$CaCO_3$ (mg L^{-1})	18.93	7.79	27.48
DOC	mg L^{-1}	1.90	0.62	NM
COD	mg L^{-1}	<DL	<DL	101
Setteable solids	mL L^{-1}	0.5	<DL	25
Aluminum	mg L^{-1}	0.43	<DL	70.68
Iron	mg L^{-1}	3.59	1.00	97.80

Notes: COD = chemical oxygen demand; DOC = dissolved organic carbon; DL = detection limit; NM = not measured; FBW = filter backwash water.

Detection of (oo)cysts from purified suspensions

The recovery efficiency (RE) obtained for each of the tested methods is shown in Table 3, in comparison to what USEPA (2012) recommends for water. Flocculation with ferric sulfate followed by purification stood up for recovering *C. parvum* from sludge, as well as MF and IMS in FBW, but none of the procedures reached satisfactory results for *G. muris*, which is further explained.

Table 3 – Protozoan recovery efficiencies (RE) of the selected methods for WTP sludge and filter backwash water (FBW) samples.

Matrix	Recovery method	*Giardia muris*			*Cryptosporidium parvum*		
		Average inoculum (cysts)	RE (%)	CV (%)	Average inoculum (oocysts)	RE (%)	CV (%)
Sludge	DC+ICN7X + IMS	1640±218	0.00±0.07	>100%	433±102	30.00±8.78	28.79
	CCF	1853±197	0.27±0.27	100.00	583±164	12.00±7.95	66.24
	CCF + IMS	1837±36	0.16±0.04	23.57	1025±243	11.27±2.01	17.85
	$Fe_2(SO_4)_3$+IMS	1491±170	6.44±2.06	31.95	1404±208	91.20±31.06	34.05
	$Al_2(SO_4)_3$+IMS	2069±49	4.24±1.18	27.80	446±104	22.98±14.96	65.10
FBW	MF	1375±218	17.36±6.87	39.58	618±228	100±49.18	47.80
	MF+IMS	2089±429	0.00%	60.98%	738±97	43.90%	22.67%
Method 1623.1 [22]		*Giardia* spp.			*Cryptosporidium* spp.		
		-	8 – 100 %	≤39	-	32 – 100 %	≤37

Notes: $Al_2(SO_4)_3$ = aluminum sulfate flocculation; CCF = calcium carbonate flocculation; CV = coefficient of variation; DC = direct centrifugation; $Fe_2(SO_4)_3$ = ferric sulfate flocculation ICN 7X = elution solution; IMS = immunomagnetic separation; MF = membrane filtration; RE = recovery efficiency.

The low recovery rates for *G. muris* may have been a consequence of the poor fluorescence of this species under FITC when labeled with Merifluor®, as described by Alderisio et al. [32], who reported how differences in staining intensities may affect cyst detection. During cyst enumeration, parallel to the inoculum, the low fluorescence exhibited by *G. muris* was noticed by the microscopist, as well as some aggregation of the organisms, as displayed by Figure 2a. It is also important to take into account that there was some debris in the slides with recovered samples, particularly because this research dealt with environmental matrices. Therefore, the fact that *G. muris* cysts were not enhanced in apple green against the background as expected [22] may have led to lower RE for this species, not necessarily representing the efficiency of the methods.

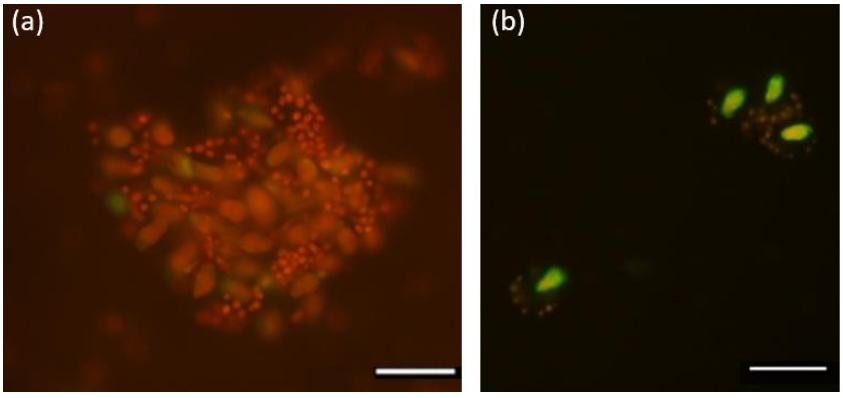

Figure 2 – *Giardia muris* cysts examined under FITC: (a) agglomerate of the purified suspension, and; (b) single cysts derived from lysis buffering. Scale bar = 20 μm.

Direct centrifugation using ICN 7X elution solution followed by IMS did not reach Method 1623.1 requirement [22] for neither target organisms. A similar research tested this protocol for (oo)cyst recovery from jar-test sludge and obtained high coefficients of variation for the target organisms (CV = 43.3% for *Giardia* spp. and CV = 85.5% for *Cryptosporidium* spp.), also not validating the method by experiments with purified suspensions [20].

As for CCF, this research also did not reach satisfactory RE, as the immunofluorescence assay was impaired by the aspect of the glass slides, which were completely opaque by the lack of sample purification. Therefore, recovery results may have been underestimated by operational difficulties derived from the matrix complexity (Table 2).

Including a purification step after CCF increased oocyst recovery, but the results were much lower than expected, based on a similar study that recovered 60.2 % of oocysts from jar-test sludge derived from simulated water with high turbidity [20]. It should be noted that the sludge from natural water has physical-chemical characteristics that differ from synthetic water. Such difference of results corroborates the need to investigate the most appropriate method for the recovery of protozoa in complex matrices.

The better results achieved for acid flocculation may indicate that the lysis buffer removes dissolved iron from the concentrate. It was first inferred because the supernatant of the centrifuged sludge samples presented a dark yellow color, which does not happen when the buffer is not applied. Secondly, the pellet reduces size after buffering with acid, as its intention is to break the flocs, thus it may also remove metal salts from the matrix itself. It is known that metal particles negatively affect the performance of IMS, which is based on the coupling of macromolecules with superparamagnetic iron oxide (Fe_3O_4) [33]. Previous studies determined that recovery levels markedly decreased in dissolved iron concentrations above 4 mg L^{-1} for *C. parvum* and 40 mg L^{-1} for *G. duodenalis* [34]. The sludge under study in this research had a 97.8 mg L^{-1} iron concentration (Table 2), which may have impaired immunomagnetic separation in the methods that did not include lysis buffering.

It is also important to point out that acid flocculation methods were the only ones that provided some *G. muris* recovery from sludge samples, which may have been caused by a disaggregation of cysts that were probably not previously available for IMS antibody attachment. As it is a selective binding procedure, the way the antibody adheres to the target organism interferes directly in the RE [35]. Microscope images have confirmed some bulking

of the cysts from the purified suspension (Figure 2a) and the effect of the acid buffer on it (Figure 2b), assessed by an exploratory test. This verification was performed by spiking an aliquot of the *G. muris* suspension into a centrifuge tube with distilled water and undertaking the same lysis procedure the sludge samples were submitted to. The effect on the cysts was analyzed by IFA and the results are shown in Figure 2b, which displays disaggregated cysts.

Regarding *C. parvum* recovery for the two flocculants tested for acid flocculation, concentrating the sludge samples with ferric sulfate met USEPA [22] minimum requirements for water both in RE and CV (Table 3), whilst the treatment with aluminum sulfate did not. The differences in RE for the acid concentration methods agree with a similar study [22] that did not include IMS for purification and obtained 61.5% recovery of *C. parvum* when ferric sulfate was applied as flocculant, whereas aluminum sulfate led to a 58.1% RE.

As for the filtration methods tested in FBW samples, the results reiterated the inferences that *G. muris* bulking might have hindered IMS performance, as MF without a purification step led to some recovery for this species (RE = 17.36%), which would have been compatible to USEPA [22] requirements for water, if not for CV higher than 39%. Cyst agglomerations are more likely to be retained by membranes, as well as their mechanical extraction and elution are much easier, due to their size. Although the purification step was expected to increase RE, antibody binding may again have been impaired by cyst bulking, causing MF followed by IMS to lead to a lower RE than membrane filtration by itself. As regards *C. parvum* recovery, MF exceeded 100% RE, which is a result derived by some imprecision of working with multiplication factors (Equation 1) and the differences in the obtained results show in the coefficient of variation (CV = 47.80%). Membrane filtration followed by IMS reached USEPA [22] requirements for oocyst recovery from water in both RE and CV. These results, although lower than MF, do not disagree with the expectations for such technique, as they provide acceptable efficiencies for water, which presents similar physicochemical characteristics to the simulated FBW (Table 2) under study.

Taking the discussed challenges of working with purified suspensions of *G. muris* into account, Table 4 presents the statistical analysis ran by the RE of *C. parvum* only. The higher RE by ferric sulfate flocculation followed by IMS was significantly different and therefore selected for analytical quality assessment. Concerning FBW, the results obtained by including the purification step also led to significant difference, thus MF followed by IMS was selected for the recovery using ColorSeed®, because its average results with the purified suspensions had reached USEPA (2012) requirements for water.

Table 4 – Statistical comparison of the selected methods for oocyst recovery (t test where $p \leq 0.05$ was considered significant).

Matrix	Recovery methods compared	*p*-value
Sludge	CCF + IMS vs DC + ICN7X + IMS	0.538
	CCF + IMS vs $Fe_2(SO_4)_3$+ IMS	0.019
	CCF + IMS vs $Al_2(SO_4)_3$+ IMS	0.763
	DC +ICN7X + IMS vs $Fe_2(SO_4)_3$+IMS	0.020
	DC +ICN7X + IMS vs $Al_2(SO_4)_3$+IMS	0.535
	$Fe_2(SO_4)_3$+ IMS vs $Al_2(SO_4)_3$+ IMS	0.019
FBW	MF vs MF + IMS	0.012

Notes: $Al_2(SO_4)_3$ = aluminum sulfate flocculation; CCF = calcium carbonate flocculation; CV = coefficient of variation; DC = direct centrifugation; FBW = filter backwash water; $Fe_2(SO_4)_3$ = ferric sulfate flocculation; ICN 7X = elution solution; IMS = immunomagnetic separation; MF = membrane filtration; RE = recovery efficiency.

Analytical quality assessment

RE obtained for both of the tested methods when ColorSeed® was inoculated in the samples are indicated in Table 5 and were validated by the USEPA (2012) requirement for water samples for *Giardia* spp.. This corroborates that *Giardia muris* poor fluorescence [32] may have influenced RE in the previous tests. Nevertheless, when *Cryptosporidium* spp. oocysts were targeted, the minimum standards were not reached, the opposite effect verified for the recovery assays when purified suspensions were spiked into the samples.

Table 5 – Analytical quality assessment of the selected methods for (oo)cyst recovery from sludge and FBW.

Matrix	Method	*Giardia* spp.		*Cryptosporidium* spp.	
		RE (%)	CV (%)	RE (%)	CV (%)
Sludge	$Fe_2(SO_4)_3$+ IMS	32.25 ±3.11	9.00	11.00±5.24	47.67
FBW	MF + IMS	13.00 ±2.55	19.61	2.00±1.87	93,54
Method 1623.1 [22]		8 – 100 %	≤ 39	32 – 100 %	≤ 37

Notes: CV = coefficient of variation; $Fe_2(SO_4)_3$ = ferric sulfate flocculation; IMS = immunomagnetic separation; MF = membrane filtration; RE = recovery efficiency.

One of the main differences between the experiments is the fact that analytical quality assays are run with ColorSeed® kits, which contain a defined number of organisms (99±1.3 *Giardia* spp. cysts and 99±1.7 oocysts of *Cryptosporidium* spp.), fewer than the average (oo)cysts spiked from suspension aliquots (Table 3). As a matter of probability, the greater the number of organisms in the matrix, the greater the chance of recovery [36]. A similar study may reaffirm this point as, when oocysts were recovered from samples by ferric sulfate flocculation (without IMS), containing $1x10^6$, $2{,}5x10^5$ and $1x10^5$ oocysts each, the achieved RE were 68.1, 61.5 and 47.0%, respectively [26].

Effects in oocysts viability

Positive results for PI inclusion in *C. parvum* oocysts according to the tested methods are shown in Table 6. Despite there were two pathogens being recovered, only *C. parvum* was targeted for viability considering the low RE obtained for *G. muris*. The data was based on the mean percentage of stained oocysts in relation those identified under FITC and this proportion was obtained for the inoculum (representing the viability state of the oocysts before they were subjected to recovery procedures), as well as for the recovered samples. Organisms that included PI are generically understood as non-viable and increases in PI-stained oocysts represent loss in membrane integrity throughout recovery.

The results indicated that all of the recovery assays affected oocysts viability. Methods that required changes in pH (calcium carbonate, aluminum sulfate and ferric sulfate flocculation) led to higher specific PI uptake, whereas membrane filtration with heated Tween® 80 with or without IMS did not exceed 30% of PI inclusion in oocyst recovered samples. It is worth pointing out that direct centrifugation with ICN 7X, as one of the least invasive methods, caused more than 90% red staining in the recovered sample. The difference proportion of PI inclusion correlated to the suspension age (r = 0.9107), which might indicate older oocysts may be more susceptible to membrane damage, associated to viability.

The detection method by the immunofluorescence assay (IFA) coupled to PI-staining in dried slides may have also affected membrane integrity, as described in Robertson et al. [37], who compared an increase in PI uptake when this vital stain was added to (oo)cysts after drying samples. In the present research, this effect is incorporated to the entire procedure of recovering and visualizing microorganisms. Nevertheless, PI staining in suspension and its possible correlation to excystation or infectivity is recommended for further research with aims of investigating viability.

Table 6 – Average effect of the recovery method on propidium iodide (PI) uptake by *C. parvum* oocysts.

Matrix	Recovery method	PI stained oocysts in the inoculum (n=3)	PI stained oocysts in the recovery assay (n=4)	Suspension age
FBW	MF	7.33%	25.34%	128 days
FBW	MF+IMS	11.34%	29.48%	134 days
Sludge	CCF + IMS	34.15%	88.53%	169 days
Sludge	DC+ICN7X + IMS	19.40%	90.53%	177 days
Sludge	$Fe_2(SO_4)_3$+IMS	33.22%	94.89%	191 days
Sludge	$Al_2(SO_4)_3$+IMS	36.10%	98.78%	204 days

Notes: Notes: $Al_2(SO_4)_3$ = aluminum sulfate flocculation; CCF = calcium carbonate flocculation; DC = direct centrifugation; $Fe_2(SO_4)_3$ = ferric sulfate flocculation; ICN 7X =

elution solution; IMS = immunomagnetic separation; n = number of repetitions that lead to the average value; MF = membrane filtration; RE = recovery efficiency.

Conclusions

This study reveals the challenges in protozoan recovery in simulated sludge samples of WTPs, as well as FBW, by testing different methods in an exploratory approach. These difficulties were mainly attributed to sediments and dissolved metals in the matrices, which reaffirms the need of a purification step during the recovery assay. However, IMS may be hindered by dissolved iron and aluminum, which are common in WTP residues, as this study has also showed. Therefore, procedures that remove these metals during the concentration step are recommended, such as using a lysis buffer after flocculation, which has increased the recovery efficiency (RE) in this research. Although RE was greater, both flocculation by ferric sulfate and membrane filtration followed by IMS were not sufficient for *Cryptosporidium* spp. recovery in the analytical quality assessment, even though they did it for *Giardia* spp., which endorses the complexity of the matrix and encourages further research. In addition, all of the tested methods have proved to affect membrane integrity of *C. parvum* oocysts, assessed by propidium iodide exclusion, indicating that recovery assays may affect viability, an important parameter to co-investigate when such organisms are targeted in public health.

Disclosure statement

The authors reported no potential conflict of interest.

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