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# Ferric sulphate flocculation as a concentration method for *Giardia* and *Cryptosporidium* in filter backwash water

Kamila Jessie Sammarro Silva and Lyda Patricia Sabogal-Paz*

Department of Hydraulics and Sanitation, São Carlos School of Engineering, University of São Paulo, Avenida Trabalhador São-carlense 400. São Carlos, Zip code: 13566-590, São Paulo, Brazil

*Corresponding author. E-mail: lysaboga@sc.usp.br

## Abstract

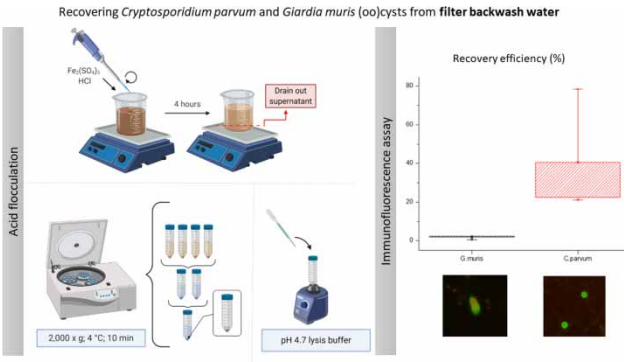
Filter backwash water (FBW) is a prominent residue from water treatment plants (WTPs) that is often disposed into water bodies or recycled within the WTP without due disinfection. FBW usually contains particles within a size range that includes pathogenic protozoa, as the infective forms of *Giardia* and *Cryptosporidium*, parasites responsible for waterborne diseases outbreaks. Quantifying (oo)cysts is essential for addressing this matter, as it might assist research on giardiasis and cryptosporidiosis epidemiology, as well as shed light onto disinfection technologies for FBW. However, (oo)cyst recovery from FBW and other complex matrices still lacks a standard protocol and entails specialized professionals and expensive material. Seeking to provide insight in a reduced-cost recovery method, this study analysed the recovery efficiency (RE) obtained by acid flocculation with ferric sulphate, a common coagulant, on bench-scale simulated FBW. Steps included concentration by flocculation, centrifugation, and quantification by immunofluorescence. Although recovery was sufficient for *Cryptosporidium parvum* (40.59%), Method 1623.1 recommendations were not reached for *Giardia muris* (1.76%). Coefficients of variation obtained for both organisms were not satisfactory, highlighting the variability to which environmental matrices are subjected and why defining a methodology for (oo)cyst recovery in WTP residues is important.

**Key words:** immunofluorescence, pathogenic protozoa, protozoa recovery, water treatment residue

## Highlights

- *C. parvum* oocyst recovery efficiency (RE) complied with Method 1623.1.
- *G. muris* RE from FBW was insufficient and statistically different from *C. parvum*'s.
- Coefficients of variation for both microorganisms were higher than Method 1623.1 limits.

## Graphical Abstract



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## INTRODUCTION

*Cryptosporidium* and *Giardia* are genera of parasitic protozoa responsible for worldwide outbreaks of enteric diseases that have been reported throughout decades (Efstratiou *et al.* 2017; Ligda *et al.* 2020). Monitoring cysts and oocysts (i.e. their infective forms released into the environment) in sources of public water supply is of major concern in order to contain waterborne transmission (Ongerth 2013). Additionally, this matter must be addressed within water treatment plants (WTPs), particularly considering a potential contamination scenario where there is inadequate management of treatment residues, as in dumping them into water bodies or recycling filter washing water into the system without proper disinfection (Freitas *et al.* 2010).

Monitoring and detecting (oo)cysts requires concentration of the samples, potentially followed by purification and, finally, a quantification assessment. In this regard, Method 1623.1 (USEPA 2012) provides guidelines for recovering *Cryptosporidium* spp. and *Giardia* spp. (oo)cysts from water samples, which allows quantifying these organisms and offers material to assess microbiological risk within water sources. However, there are inherent analytical challenges associated with the detection of these pathogens (Ligda *et al.* 2020). In addition, some matrices such as filter backwash water (FBW) differ from water samples in physicochemical properties, as those derived by naturally occurring debris from raw water (e.g. clays, algae organic material, etc.), coagulant residuals (particularly dissolved metals and polymers), among others, that directly interfere in turbidity and pH. These may lead such matrices to an incompatibility with Method 1623.1 protocol (USEPA 2012), which is also costly and requires specialized professionals (Ligda *et al.* 2020). All of these make research in alternative recovery methods timely, especially when considering places where neglected tropical diseases are predominant.

This methodology gap encourages investigation into alternative and matrix-specific recovery assays, among which flocculation techniques (Karanis & Kimura 2002; Kourenti *et al.* 2003) may stand out, due to their reduced cost in comparison to Method 1623.1 (Andreoli & Sabogal-Paz 2017). Ferric sulphate flocculation, specifically, has shown satisfactory recovery efficiencies in high-turbidity samples of water treatment sludge (Sammarro Silva & Sabogal-Paz 2020), highlighting it as a potential method when considering similar matrices.

Therefore, this study aimed to assess the (oo)cyst recovery efficiency of ferric sulphate flocculation as a lower-cost concentration method for WTP liquid residue. The matrix under test was bench-scale simulated filter backwash water artificially contaminated with *Giardia muris* cysts and *Cryptosporidium parvum* oocysts.

## METHODS

Water from Monjolinho River, a surface water source located in the municipality of São Carlos (São Paulo State, Brazil), was used as raw water for the treatability tests performed in this study. Complete jartest runs (i. e. including filtration) were carried out to assess treatability. Polyaluminium chloride (PACl 17.51% $Al_2O_3$) worked as a coagulant under optimal conditions, tested by turbidity removal considering a 5–15 mg $L^{-1}$ dosing range and pH interval from 6 to 10 (adjusted by adding either sulfuric acid or sodium hydroxide, both purchased from Sigma-Aldrich®). Operational conditions were fixed: rapid mixing under a gradient of 1,000 $s^{-1}$ for 10 s; slow mixing under 30 $s^{-1}$ for 20 min; settling velocity of 2.0 cm $min^{-1}$.

The laboratory filters coupled to the jartest equipment consisted of graduated acrylic tubes with 19 mm internal diameter and a metallic mesh as support. Filtering medium consisted of sand with size ranging from 0.30 to 0.59 mm. This bench-scale filtering was performed under a 100 $m^3\ m^{-2}\ d^{-1}$ maximum rate (Maciel & Sabogal-Paz 2016), which means that, within the period of 60 s,

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approximately 20 mL of filtered water should be produced. This was confirmed regularly by timed volume verifications. As well as for decanted water, the water quality of filtered water was also evaluated, in order to assess if the simulated treatment would be representative.

After a series of complete jartest runs with filtration of approximately 350 mL of decanted water (2.5 min stabilization plus approximately 15 min filtering), FBW samples were obtained by three subsequent washes. Filter washing was performed by using a 50 mL syringe filled with deionized water upflow. The matrix under study was characterized considering physical and chemical parameters, also tested for the raw water and treated samples. These were pH (Digimed® DM-20 pHmeter), turbidity (HACH® 2100 P turbidimeter), apparent colour (Digimed® DM-COR portable colour meter) and dissolved iron (fast sequential atomic absorption – AA 240 FS, Method 3111B) (APHA *et al.* 2012).

FBW samples were artificially contaminated by aliquots of purified *Cryptosporidium parvum* oocysts and *Giardia muris* cysts from a commercial source (Waterborne™, Inc). According to the suppliers, oocysts and cyst suspensions resulted from Percoll and sucrose density gradient centrifugation of faeces of experimentally infected calves and Swiss Webster mice, respectively. Aliquots were spiked into 500 mL samples of filter backwash water ($n = 4 +$ blank control) and an estimation of the (oo)cysts input was obtained from a duplicate count of the inoculum, concomitant to the recovery assay. All vessels and materials that were exposed to the protozoa were previously rinsed with Tween® 80 elution solution (0.1% v $v^{-1}$).

Recovery tests were performed using flocculation by ferric sulphate ($Fe_2(SO_4)_3$) (Karanis & Kimura 2002). The flocculant was added in order to obtain a final $Fe^{3+}$ concentration of 5 mg $L^{-1}$ in the 500 mL FBW samples. pH was adjusted to 6.0 by hydrochloric acid and the samples were kept standing for 4 hours prior to careful removal of approximately 75% of the supernatant. The concentrated product was centrifuged (2,000 × g; 4 °C; 10 min) and a 1 mL pellet was obtained. The latter was vortexed with 1 ml of lysis buffer (citric acid monohydrate and sodium citrate buffered to pH 4.7) and left to settle for 60 min. Three ultrapure water washes were performed and aliquots of 100 μL (divided into two 50 μL microscopy wells) of each 1 mL final sample had their (oo)cyst counted, leading to a 10-fold multiplication factor.

Cyst and oocyst quantification was performed by immunofluorescence assay (IFA) using the Merifluor® kit and morphological aspects were observed by 4′6-diamidino-2 phenylindole (DAPI) staining. Samples were examined by an epifluorescence microscope under 400× magnification (BX51, Olympus®).

Data analysis was carried out using Origin® 6.0 software for descriptive and inferential statistics. Sample probability distribution was verified prior to applying statistical analysis, by Shapiro-Wilk normality test under a 95% confidence interval and outliers were also verified by Grubb's test under the same significance level. Results were compared to the minimum expected RE for each pathogen, outlined in Method 1623.1 from USEPA (2012), as the matrix under study lacks a specific protocol. The recovery efficiencies of the two target organisms were also tested for the central hypothesis of equal means among themselves.

## RESULTS AND DISCUSSION

Raw water average turbidity was 30.8 ± 5.2 NTU and its apparent colour was 76.6 ± 9.9 HU with a 7.54 ± 0.01 pH ($n = 30$ collections for jartest runs). Figure 1 displays the coagulation diagram obtained for different PACl dosing, suggesting 10 mg $L^{-1}$ as an optimal working concentration. Considering the 93.9% turbidity removal obtained in the decanted water when no pH changes were performed, this condition (black arrow in Figure 1) was adopted for the following-up complete jartest assays.

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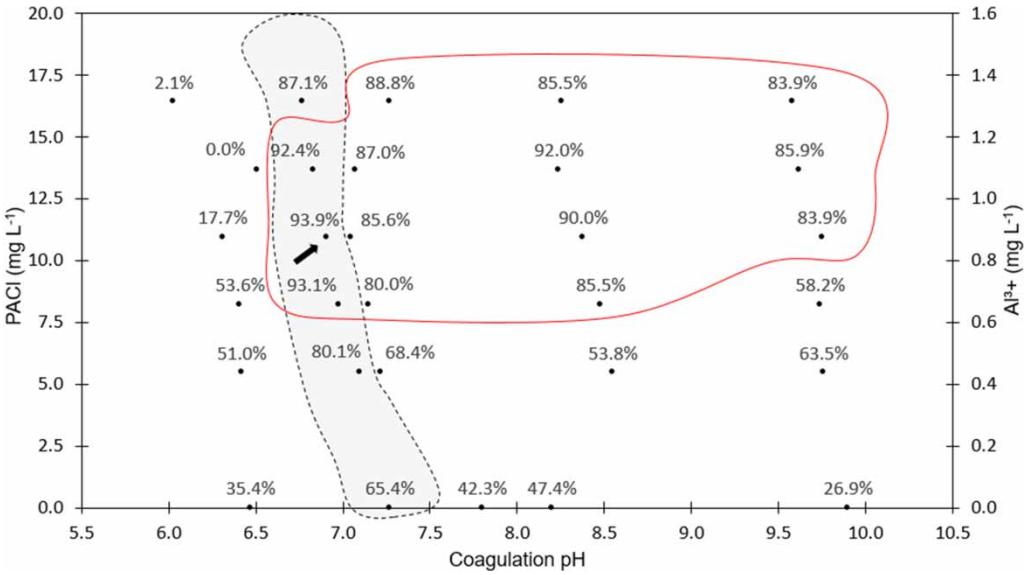


**Figure 1** | Coagulation diagram for turbidity removal using polyaluminum chloride (PACl) after coagulation at 1,000 $s^{-1}$ for 10 s and slow mixing under 30 $s^{-1}$ for 20 min. Settling velocity was fixed at 2.0 cm $min^{-1}$. Red continuous line indicates reminiscent turbidity lower than 5 NTU. The shaded region within the dashed line highlights results without change in initial pH. The arrow points to the highest turbidity removal.

Bench-scale simulation of the complete water treatment set was considered successful, as filtered water samples led to results under the detection limit for apparent colour and final turbidity below 0.5 NTU. Figure 2 displays the evolution of reminiscent turbidity throughout the treatment that leads to effective clogging of filtering media in order to provide the desirable qualities for washing residue to be evaluated for protozoan recovery.

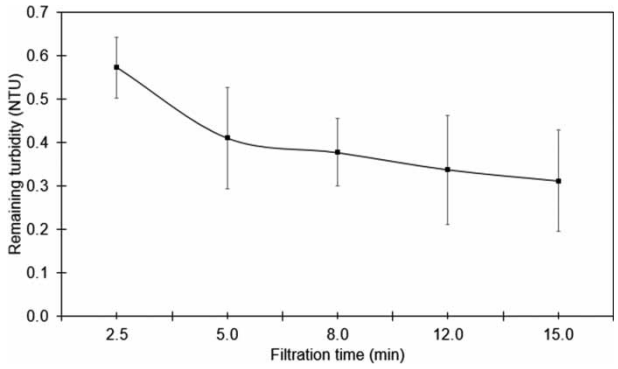


**Figure 2** | Remaining turbidity throughout bench-scale filter runs, considering 2.5 min for stabilization. Error bars indicate the standard deviation for multiple collections ($n = 3$).

Concerning such residue, filter backwash water (FBW) presented 6.62 pH, turbidity of 6.6 NTU and 8.5 HU for apparent colour, obtained by compound samples, into which (oo)cysts were spiked. Concerning dissolved iron concentration, surface water contained 3.593 mg $L^{-1}$, whilst FBW presented 1.003 mg $L^{-1}$.

As recovery data was normally distributed for both parasites (Shapiro-Wilk normality test, considering $p < 0.05$), Figure 3 displays recovery efficiency (RE) results for the two target organisms within a boxplot representation. Similarly, Table 1 indicates descriptive statistics in terms of (oo)cyst counts.

Recovery efficiency did not meet the Method 1623.1 RE requirement (USEPA 2012) for *Giardia* spp., which is 8–100% for water samples. This inference was corroborated by a one-sample Student's

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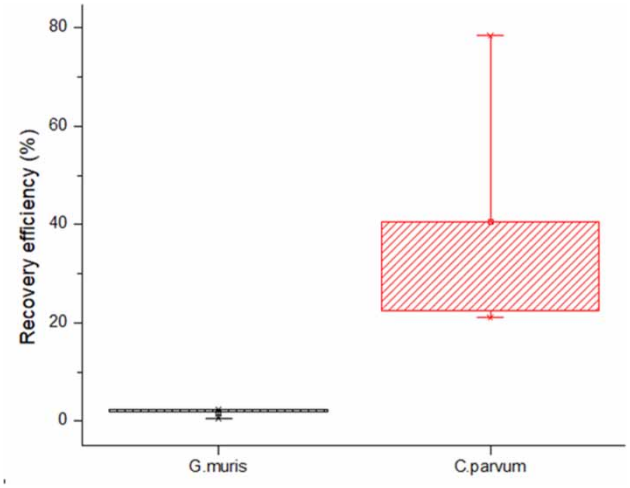


**Figure 3** | Boxplot display of recovery efficiencies (percentage) of *Giardia muris* and *Cryptosporidium parvum* artificially spiked into simulated filter backwash water (FBW) using ferric sulphate flocculation as a concentration method.

**Table 1** | Recovery of (oo)cysts from filter backwash water (FBW) using ferric sulphate flocculation

| Sample | *Giardia muris* | *Cryptosporidium parvum* |
|---|---|---|
| 1 (number of (oo)cysts) | 4 | 56 |
| 2 (number of (oo)cysts) | 1 | 16 |
| 3 (number of (oo)cysts) | 5 | 15 |
| 4 (number of (oo)cysts) | 5 | 29 |
| Average number of (oo)cysts | 3.75 ± 1.64 | 29.00 ± 16.54 |
| Mean RE (%); CV (%) | 1.76; 43.72 | 40.59; 57.03 |
| Method 1623.1 (USEPA 2012) recovery (%); CV (%) | 8–100; ≤39 | 32–100; ≤37 |

*Notes*: Average inoculum of *G. muris* was estimated as 2,130 ± 211 cysts (10 µL suspension aliquot, $n = 2$) and *C. parvum* 714.4 ± 24.5 oocysts (10 µL suspension aliquot, $n = 2$) in 500 mL of FBW. Mean recovery based on the multiplication factor. CV = coefficient of variation, RE = recovery efficiency.

*t*-test against the hypothetical mean of the minimum percentage RE from Method 1623.1 ($p < 0.0001$). As for *Cryptosporidium* spp., the 32–100% recovery was reached (40.59%; $p = 0.5662$ for equal means), but the technique did not provide a coefficient of variation (CV) under 37%.

Recovery efficiencies found in this research were lower than reports in literature, which mentioned ranges from 47 to 68% for *Cryptosporidium parvum*, as a function to the number of spiked organisms (Karanis & Kimura 2002). The paper that described this methodology applied tap water and varied orders of magnitude ($10^5$–$10^6$) for the number of seeded oocysts per litre (Karanis & Kimura 2002). Those RE results have complied with Method 1623.1 (USEPA 2012) requirements for water, which were not met in the present study when *Giardia muris* was targeted, but did for *C. parvum*. As of orders of magnitude, our research considered an average inoculum of $10^3$ organisms per litre of filter backwash water (FBW) and the greater the occurrence, the most likely it is to recover the organisms (Assavasilavasukul *et al.* 2008). Yet, this incongruence also suggests matrix influence in RE.

Ferric sulphate has also been studied for concentrating protozoa from simulated water treatment sludge (Sammarro Silva & Sabogal-Paz 2020); however, as the matrix under test was of high turbidity and solid content, the recovery process included purification; that is, an additional procedure aiming at selectively concentrating target organisms from the particle assemblage. Although sludge and FBW differ in quality parameters, it is important to point out that, even among research contemplating the same type of matrix, physicochemical characteristics vary. The reason for that relies on the fact that

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even though different water treatment plants may operate with the same technology, it does not necessarily end in the same effluent quality, considering factors such as filter media, operation, maintenance, and raw water quality (Melo *et al.* 2019). Likewise, water treatment residues will vary in characteristics, which also encourages studies with real matrices, particularly considering reports of (oo)cysts retained in pilot-scale filters (Hsu & Yeh 2003) and data on contamination of backwash water from full-scale rapid filters (Karanis *et al.* 1996).

Illustratively, it is possible to mention a work with bench-scale simulated water treatment residue, in which authors used kaolinite and humic acid for their study water. Their filter backwash water presented turbidity of 26.7 NTU and pH 6.1 (Silva & Sabogal-Paz 2020), similar properties to those obtained in this research in terms of pH, but rather higher in turbidity (6.62; 6.6 NTU). Nonetheless, artificial-based raw water often lacks metals, as in iron concentration was less than 0.1 mg $L^{-1}$ in the cited work (Silva & Sabogal-Paz 2020), whereas this study presented 1.003 mg $L^{-1}$, likely to be derivative from the surface water source. Dissolved metals such as iron and aluminium may influence recovery during both concentration and purification steps (USEPA 2012). Concentration methods based on flocculation rely, among other mechanisms, on salt precipitation (Vesey *et al.* 1993). Thus, the concentrate may well vary in features according to matrix quality, which possibly will either allow or hinder analysis of the entire pellet. Accordingly, a study on *Cryptosporidium* and *Giardia* occurrence in real filter backwash water from a water treatment plant applied calcium carbonate flocculation to concentrate its samples justified by the metal-based coagulant used beforehand (Ladeia *et al.* 2018). Although not performed in the present paper, the efficacy of immunomagnetic procedures for purification may be markedly affected by metals, as presented in a research aimed at recovering *C. parvum* oocysts from solutions containing various concentrations of dissolved iron (Yakub & Stadterman-Knauer 2000).

Other studies have considered membrane filtration (MF) for concentrating filter backwash water (Sammarro Silva & Sabogal-Paz 2020), as well as matrices similar in quality parameters (medium turbidity) as in treated wastewater effluents (Medeiros & Daniel 2015). The former led to cyst RE of approximately 17.4% and over 100% for oocysts with high standard deviation, whereas the latter found a 35% recovery for *Giardia* spp. and 12.5% for *Cryptosporidium* spp., displaying a wide variability range. Here we tested concentration by ferric sulphate flocculation, which is an acid-based flocculation technique and found coefficients of variation (CV) that did not comply with the established recommendations from Method 1623.1 (USEPA 2012). Although flocculation is low-priced when compared to MF (Maciel & Sabogal-Paz 2016; Andreoli & Sabogal-Paz 2017), its efficiency must also be assessed in different conditions, as the matrix quality parameters vary, causing protozoan cysts and oocysts to possibly fail to settle during concentration or, on an opposite prospect, cause the concentrate to be incompatible with purification or detection steps.

As for increasing recovery efficiency in general terms, adding a purification step may be an option. Immunomagnetic separation (IMS) is a technique that has stood out in various water and wastewater matrices (Hsu & Huang 2000; McCuin & Clancy 2005) and has been endorsed by Methods 1623.1 and 1693 (USEPA 2012, 2014). Selective concentration by density gradient techniques, listed for further information in a fairly recent review of recovery methods (Efstratiou *et al.* 2017), could be an alternative for situations in which IMS costs renders its application impractical.

The high CV in protozoa research lie on the fact that (oo)cyst recovery has an inherent high variability (Francy *et al.* 2004), as only a fraction of the present organisms are recovered and analysed from a selected volume (Ongerth 2013) and organisms are not distributed uniformly, particularly in low concentrations (Ongerth & Saaed 2013). Although there were no outliers within the confidence interval established for this research ($\alpha = 0.05$), more repetitions would be an option for reducing data variability. Nonetheless, costs associated with the detection step by immunofluorescence assay (IFA) often limit repetition, particularly in developing countries (Maciel & Sabogal-Paz 2016). This effect on the coefficient of variation has also been reported in similar research on FBW (Silva &

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Sabogal-Paz 2020), in which recovery assays by direct centrifugation with ICN 7X cleaning solution (MPBio®) and subsequent purification led *Giardia* spp. RE ($n = 4$) to reach Method 1623.1 requirements, but a CV of 82% for *Cryptosporidium* spp. A study on formalin/ether concentration method as a low-cost alternative (Lora-Suarez *et al.* 2016) has also mentioned the need of assessing effects of turbidity on RE, as well as performing interlaboratory comparisons to test reproducibility. Besides these points, high standard deviations may be attributed to deficiencies in counting methods (Kourenti *et al.* 2003), different (oo)cyst strains and storage media (Karanis & Kimura 2002), as well as propagation of imprecisions by applying multiplication coefficients when the concentrate is not entirely analysed (Sammarro Silva & Sabogal-Paz 2020).

A Student's *t*-test indicated that the mean recovery for *G. muris* and *C. parvum* was significantly different ($p = 0.0272$; $\alpha = 0.05$; Figure 3). This suggests there are different efficiencies among microorganism's recovery from filter backwash water using ferric sulphate flocculation as a procedure for concentrating samples. Concerning this statistically significant difference found when pairing both target organisms, we assume that the lower *G. muris* RE may be explained by its poor fluorescence when observed by immunofluorescence using commercial kits for detection (Alderisio *et al.* 2017). In other words, the low fluorescence intensity presented by this species may have affected RE, as some recovered cysts may not have been detected and accounted for. This matter has also impaired analogous research into quantity assessment (Sammarro Silva & Sabogal-Paz 2020). Thus, fluorescence intensity should be considered when using *G. muris* cysts as contamination models for *Giardia* spp., regardless of their similar morphologic features and resistance to treatments (Finch *et al.* 1993; Mofidi *et al.* 2002) if identification is undertaken by immunofluorescence assays.

## CONCLUSIONS

This study reveals the recovery efficiency (RE) of *Giardia muris* and *Cryptosporidium parvum* (oo) cysts from artificially contaminated filter backwash water (FBW) generated by bench-scale treatment using a natural surface water source. Ferric sulphate flocculation was applied for concentrating samples and results (40.59%) complied with Method 1623.1 minimum requirement for *Cryptosporidium* spp. RE from water samples (32%), pointing it as a prospective reduced-cost method for concentrating FBW for oocyst quantification. However, *G. muris* recovery (1.76%) was insufficient and there was a significant difference between RE for both microorganisms ($p = 0.0272$; 95% confidence interval). This ratifies the assumption that factors as physicochemical quality of the matrix and the target parasite influence recovery efficiency and real matrices should also be considered in future work. The high coefficients of variation (57% for *C. parvum* and 44% for *G. muris*) encourage further research into effective and accessible recovery methods for complex matrices, particularly due to the infectious and resistant features of (oo)cysts.

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## DISCLOSURE STATEMENT

The authors report no potential conflict of interest.

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## DATA AVAILABILITY STATEMENT

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

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