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Detection and alkaline inactivation of *Cryptosporidium* spp. oocysts and *Giardia* spp. cysts in drinking-water treatment sludge

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Abstract

Removing and inactivating *Cryptosporidium* spp. oocysts and *Giardia* spp. cysts are a challenge for drinking-water treatments, mainly because of their small sizes and resistance to chlorination. Moreover, protozoan detection methods are expensive and subject to high variability and low reproducibility, especially in high turbidity water. This research aimed at detecting and inactivating cysts and oocysts in drinking-water treatment sludge. In jar test treatability assays, 110 NTU study water was treated with polyaluminium chloride to obtain the sludge. The chosen method for detecting cysts and oocysts was direct centrifugation with ICN 7X solution followed by the immunomagnetic separation (IMS) with two acid dissociations. The inactivation of cysts and oocysts was identified by the inclusion of propidium iodide as a vital dye when the cell walls were damaged. This method's analytical quality control, which was performed with the EasySeed® suspension, obtained recovery of 3.3 ± 2.0% and 24.8 ± 8.0% for oocysts and cysts, respectively. Besides, the *Giardia* spp. recovery met the standards recommended for water by the USEPA Method 1623.1. Magnetic microspheres were found attached to the protozoan in the microscope slides after IMS, indicating some limitations of this purification method. The alkaline treatment tested a dose of 27mg CaO/100mL for 3 and 5 days, obtaining inactivation of 1.85 and 3.0 log for oocysts and 2.05 and 2.14 log for cysts, respectively. In this context, the alkaline treatment may be feasible to reduce the microbiological risk in water treatment sludge, considering an economic assessment.

Keywords

Immunomagnetic separation; polyaluminium chloride; protozoan; propidium iodide; calcium oxide; drinking-water treatment sludge.

1 Introduction

Oocysts of *Cryptosporidium* spp. and cysts of *Giardia* spp. are protozoan infective forms, which cause cryptosporidiosis and giardiasis, respectively. Although these diseases may be asymptomatic, common manifestations are diarrhoea and dehydration [1]. Cysts and oocysts have high environmental resistance [2,3], and low infectious dose (1 to 10 organisms) in humans [4,5]. *Giardia* spp. cysts can survive in water for two months at 8 °C [6], while *Cryptosporidium* spp. oocysts can remain infectious for six months at 0 to 20 °C [7,8].

In Brazil, water treatment plants (WTPs) generally use conventional technology and decantation for high turbidity water. In WTPs, most oocyst removal occurs in decantation and filtration stages [9]. Regarding aluminium coagulants, polyaluminium chloride (PACl) has been used in WTPs instead of aluminium sulphate ($Al_2(SO_4)_3$), as the former produces fewer residues without demanding pH corrections [10,11]. The WTPs generate water treatment sludge (WTS) or alum sludge as a by-product, commonly dewatered and disposed of in landfills [12]. However, there are sustainable approaches for WTS reuse (e.g. metal recovery and agricultural application) [12], which reinforces the relevance of protozoan detection and disinfection in this matrix.

In general, (oo)cysts detection is performed in three steps: concentration, purification, and protozoan visualization/counting. However, these methodologies for aquatic matrices are still subject to high variability and low reproducibility [13–17]. Methods for evaluating protozoan in water samples must meet the analytical quality control standard, proposed by the Method 1623.1 [18]. EasySeed® suspensions can evaluate initial precision and recovery for each studied matrix, with a known number of (oo)cysts. However, complex matrices (including WTS) still require standards for validating protozoan detection protocols.

Efstratiou et al. [19] surveyed the main concentration methods: filtration methods, which include membranes, ultrafiltration, microfiber, and nanofiltration; flocculation-sedimentation with calcium carbonate ($CaCO_3$), $Al_2(SO_4)_3$, ferric sulphate ($Fe_2(SO_4)_3$, and formaldehyde with ethyl acetate or ether; and direct centrifugation (DC) methods batch or continuous flow. The calcium carbonate flocculation (CCF) method is recommended for high turbidity water because of the lower-cost procedures [13,14,20,21]. However, its limitations are the loss of organisms in the supernatant, reading errors due to particulates, and pH changes [14,17,22].

The DC method concentrates particles in a pellet, usually performed for larger volumes. Boni de Oliveira [23] obtained greater cleaning wells by applying the detergent dispersion solution ICN 7X 1.0% after centrifugation of soil samples, with 37.60 and 33.2% recoveries for 500 and 1000 *Giardia* spp. cysts, respectively. Giglio and Sabogal-Paz [15] evaluated protozoan detection methods on WTSs, whereas the CCF presented higher recoveries of 68 ± 17% for *C. parvum* oocysts and 42 ± 7% for *Giardia* spp. However, the DC + ICN7X method had less interference in protozoan viability in comparison to CCF, $Al_2(SO_4)_3$, and $Fe_2(SO_4)_3$ protocols [16].

The purification step is performed by density gradient, cytometry or immunomagnetic separation (IMS) [19]. Among these methods, IMS consists of (oo)cyst attachment on magnetic microspheres. Then, it is followed by the dissociation step, which is a process for detaching (oo)cysts, usually by acid or thermal dissociation. However, the WTPs sludge (WTS) usually has significantly higher turbidity, a challenge for protozoan detection methods [14]. *C. parvum* oocysts recovery can reduce drastically in higher turbidity matrices by applying the IMS [24,25]. For recovering *Cryptosporidium* spp. oocysts by DC followed by IMS method, Ryu et al. [26] achieved efficiency between 79.2 and 88.0% for low turbidity water samples, and 33.1 % for high turbidity.

For evaluating viability, the animal infectivity method is the most reliable, however this procedure is unfeasible regarding costs, applicability, and ethical issues [27]. Many studies have used vital dyes as propidium iodide (PI) with DAPI (4',6-diamino-2-phenyl-indole) and fluorescein isothiocyanate (FITC). Vital dyes can be used to identify viable organisms when there is no incorporation into the microorganisms, indicating that the cell wall has not been ruptured [28]. Campbell et al. [29] observed that the PI method showed a correlation with viability by the excystation method for oocysts of *Cryptosporidium* spp. The overestimated value was an advantage for safety. Also, Smith and Smith [30] compared PI with fluorescein diacetate for *G. intestinalis* cysts and concluded that PI had a correlation with in vitro excystation assays. Furthermore, Olson et al. [3] obtained a correlation between rat infectivity tests and PI viability, considering water and complex matrices (soil and cattle faeces).

Alkaline treatment of WTSs consists of adding an alkalinizing compound to increase pH, thus breaking down microorganism cell walls. Thermal and alkaline treatment reduces sludge water percentage, minimizing costs incurred by disinfection and final disposal [31]. Although less research has been carried out on WTS [17], there are many studies on wastewater sludge (WWS) alkaline treatment. Bean et al. [32] applied calcium hydroxide for WWS treatment, raising pH to 12, thus inactivating *Giardia lamblia* cysts, while *C. parvum* oocysts remained viable in up to 72 h of experiments. Capizzi-Banas et al. [33] inactivated helminth eggs, which are resistant bioindicators, with a pH greater than 12, a temperature of 60ºC with a contact time of 8 min. Allievi et al. [34] obtained better disinfection when using ammonium hydroxide solution than potassium hydroxide solution for treating WWS on pH 10, with faecal coliforms as indicators.

Monitoring protozoan in Brazilian supply sources is still scarce, and these analyses are costly and operationally difficult [22]. Franco (2007) pointed out the underreported protozoan contamination in Brazil, mainly due to a lack of research and monitoring [35]. Proper water

resources monitoring is crucial since sewage collection and treatment services are inefficient, and the intensification of agricultural activities may contribute to faecal contamination [36,37]. Moreover, oocysts also present higher soil survival potential than cysts [3], which may risk runoff contamination to water bodies. In this scenario, besides (oo)cyst detection, evaluating viability contributes to estimating disinfection efficiency for safely disposing of this residue.

This paper aimed at detecting and inactivating *Giardia* spp. cysts and *Cryptosporidium* spp. oocysts in the WTS. Inactivation was performed by the alkaline treatment with calcium oxide (CaO), and the challenges on protozoan detection and disinfection in high turbidity matrices were highlighted. Moreover, the inactivation of (oo)cysts was identified by the inclusion of PI as a vital dye for damaged cell walls. Besides, the procedure's costs were estimated, which is a relevant aspect of selecting detection protocols and disinfection technologies in developing countries.

Materials and Methods

This research's experimental stages consisted of preparing WTS matrices, validating (oo)cyst concentration and detection methods, and performing the alkaline treatment with CaO. The first step included study water preparation by adding 0.16 g L^{-1} kaolinite (*Sigma-Aldrich®/Fluka* 60609) to non-contaminated groundwater, in which turbidity was approximately 110 NTU. The second step consisted of performing jar test treatability assays to obtain the WTS samples (Figure 1). The PACl used had a specific weight of 1,362 g L^{-1} and 16.68% content of Al_2O_3. Treatability parameters (coagulant dosage, coagulation pH, mixing conditions, sedimentation time) were optimized based on similar study water from Giglio and Sabogal-Paz [15]. These selected parameters achieved the highest efficiency

regarding the turbidity and colour of filtered water. The jars were filled with 2 L of study water, and the treatment performed automatically (fast-mixing of 1000 s^{-1} for 10 s and slow-mixing of 25 s^{-1} for 30 min). Then, the decantation time was 10 min and 30 s. After clarified water removal, the remaining volume was considered the WTS (approximately 80 mL). Finally, 70 mL of deionized water was used for washing each jar, and this volume was included in the WTS. Physical, chemical and microbiological analyses of the matrices were evaluated according to APHA [38].

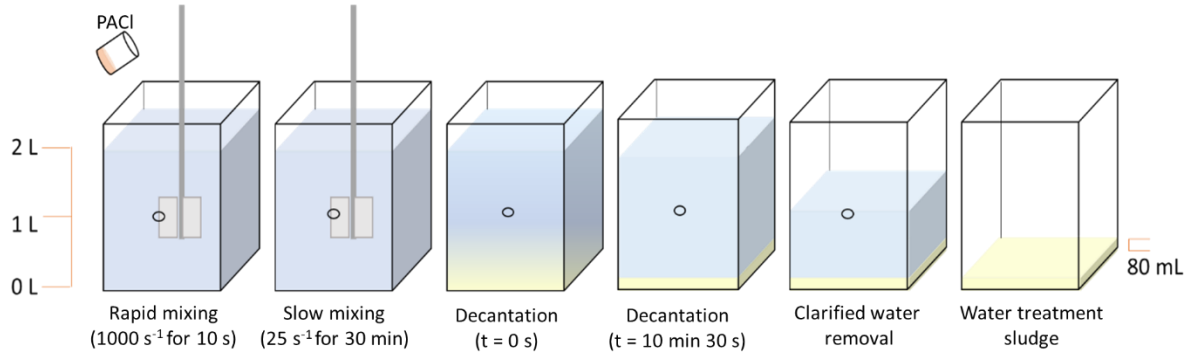

Figure 1 – Representative scheme of the jar test treatability assays, considering optimized parameters for PACl as coagulant and 2 L study water.

The commercial suspensions used were: *Giardia* spp. cysts suspension purified at the Laboratory of Protozoology, at the State University of Campinas (Unicamp, Brazil); and *Cryptosporidium* spp. oocysts suspension from Waterborne® (New Orleans, USA). All material used was previously washed with a 0.1% Tween 80 solution due to the tendency to adhesion the (oo)cysts to solid surfaces [39]. Protozoans suspensions were homogenized before each use. For counting the average number of (oo)cysts, 5 µL of both protozoan suspensions were applied to each well (n = 3). After drying overnight, the microscope slides were prepared following procedures from the Merifluor® kit (Meridian Bioscience, Inc.) and DAPI solution (Sigma-Aldrich®, F6057). According to the incorporation of PI (Sigma-

Aldrich®, P4170), the viability of protozoan in the (oo)cysts was evaluated. The volume of PI solution (10 µL) was equivalent to the suspensions added. When (oo)cysts were not stained (negative for PI dye), they were considered potentially viable.

A preliminary analytical quality assay (n = 3) with protozoans' commercial suspensions was performed for 100 mL of WTS. The detection method selected was DC + ICN 7X, followed by IMS. The 7X solution (ICN Pharmaceuticals Inc®) is neutral and phosphate-free and is non-toxic for sensitive organisms. The aimed initial concentration was ± 2000 (oo)cysts L^{-1}, which corresponded to 20 µL of the *Giardia* spp. and *Cryptosporidium* spp. suspensions. To reduce losses of target organisms throughout water treatability assays, (oo)cysts were inoculated directly into the WTS, and the sample was homogenized with a magnetic stirrer for 20 min. The volume was then divided into 50 mL Falcon® tubes (n = 2), and the centrifugation was carried out (at 1500xg for 20min). The resulting pellet (0.5 mL) from both tubes was transferred to one, besides three 0.5 mL washes with 0.1% Tween 80. After another centrifugation, the supernatant was removed, and 5 mL remaining volume was transferred to a flat-sided tube (FST), in addition to three 1 mL washes with 1.0% ICN 7X. The FST was homogenized in a rotary agitator at 20 rpm for 1 h. Then, the content was transferred to a Falcon® tube for the last centrifugation. The supernatant was removed, and 5 mL residual volume was used for the IMS step, followed by two acid dissociations, followed by the kit Dynabeads™ GC-Combo (IDEXX®).

The analytical quality control of the DC + ICN 7X and IMS protocol was validated using EasySeed® (BTF Bio – Australia), whose suspension had 99 ± 1.6 oocysts of *Cryptosporidium* spp. and 100 ± 1.9 cysts of *Giardia* spp. Five samples were processed under the same conditions, one of them a negative control (blank) to verify the absence of cross-contamination. Since there is currently no standard to be followed to validate detection protocols of (oo)cysts in WTS, the USEPA Method 1623.1 [18] was used as guidance.

For the alkaline treatment, the mass was 27 mg CaO (Sigma-Aldrich®, 248568), which corresponded to 10% of the total fixed solids (TFS) of the WTS sample. For each replicate (n = 4), the target number of (oo)cysts was approximately 5000 (oo)cysts L^{-1} on 100 mL of the WTS. Two tests simulated the alkaline treatment and drying in tropical conditions, performed in a temperature-controlled oven for 3 and 5 days at 25 ºC. After the centrifugation steps in the DC + ICN 7X method, residual pellets had volumes higher than 0.5 mL, thus not compatible with the IMS protocol. For each treatment and replicate, the samples were homogenized, divided into two centrifuge tubes, and one of them randomly selected for IMS processing. Therefore, the recovery rate considered a multiplication factor (2), due to the high cost of the Dynabeads® kit. The inactivation was calculated according to Equation 1 (where I: inactivation log of (oo)cysts; N: average number of (oo)cysts not stained after the alkaline treatment; No: average number of non-stained (oo)cysts initially). The relative number of inactivated (oo)cysts was expressed in terms of log reduction, in which every log unit corresponds to a 10-reduction factor.

$$I = -\log\left(\frac{N}{No}\right) \qquad \text{(Equation 1)}$$

The Shapiro-Wilk normality test was performed to evaluate whether the data were parametric, and parametric statistics transformed non-normal data. Then, the data were submitted to analysis of variance (ANOVA) and Student's t-test, with 95% confidence, to evaluate statistical differences of the treatments (p-value < 0.05).

Finally, the prices of the Merifluor®, Easyseed® and Dynabeads® kits were taken into consideration. Besides, alkaline treatment costs were estimated based on Brazilian Water Treatment Plants, considering a flow rate of 100 $L.s^{-1}$. The final costs were updated for April

2020, according to the General Market Price Index (IGP-M) from the *Fundação Getúlio Vargas* and considering 1 Brazilian real equal to 0.20 American dollars as of April 2020.

Results and Discussion

Groundwater presented total alkalinity of 15.3 mg $CaCO_3$ L^{-1}, conductivity of 33.2 μS cm^{-1}, turbidity of 0.2 NTU, apparent colour of 0.0 HU, pH of 6.78 and temperature of 24.3ºC. For study water, after adding 0.16 g L^{-1} kaolinite, the average values were 112 NTU turbidity, 114 HU apparent colour, 3.3 HU true colour, 8.8 mg $CaCO_3$ L^{-1} total alkalinity and 53.2 μS cm^{-1} conductivity. The filtered water presented 0.32 NTU, 0.5 HU apparent colour, and 0.0 true colour. The procedure for preparing the study water eliminated interferences that may exist in natural samples (e.g., organic matter, metals, and other microorganisms) since the objective was to evaluate the efficiency in the (oo)cysts detection and inactivation. Regarding different characteristics, natural water samples should have their treatability parameters optimized, and thus their (oo)cyst detection protocol validated [14]. The characterization of the study water, WTS and the treatability parameters are shown in Table 1.

Table 1 - Study water and WTS characterization and treatability parameters obtained in jar test bench testing (optimum conditions)

Water quality characterization	Study water	WTS	Treatability parameters	Values
Turbidity (NTU)	112	3187	PACl dosage (mg L^{-1}/mg Al^{3+} L^{-1})	25/2.2
Apparent colour (HU)	114	195	pH coagulation	6.88
True colour (HU)	3.3	2.6	Average velocity gradient in rapid mixing (s^{-1})	1000
pH	7.15	7.11	Rapid mixing time (s)	10
Zeta potential (mV)	-24.03	-9.60	Average velocity gradient in slow mixing (s^{-1})	25
Conductivity (μS cm^{-1})	53.2	54.66	Slow mixing time (min)	20

Absorbance (λ 254 nm)	0.07	0.02	Sedimentation velocity ($cm.min^{-1}$)	1.5
Alkalinity (mg $CaCO_3$ L^{-1})	8.8	6.7	Filtration rate ($m^3m^{-2}d^{-1}$)	100
Total solids (mg L^{-1})	NM	2680	Notes:	
Total volatile solids (mg L^{-1})	NM	40	NM: not measured.	
Total fixed solids (mg L^{-1})	NM	2640	< DL: less than detection limit.	
Sedimentable solids (mL L^{-1})	0.1	2.5	[1]: 150 mL of WTS for each jar	
TOC (mg L^{-1})	1.63	0.39	[2]: multiple treatability tests were performed	
COD (mg L^{-1})	<DL	3.2	for obtaining a compound sample.	
Total aluminium (mg Al L^{-1})	0.32	14960		
Total iron (mg Fe L^{-1})	0.232	1128		
Total manganese (mg Mn L^{-1})	<DL	2.1		
Total coliforms (CFU/100mL)	16	0		
Escherichia coli (CFU/100mL)	0	0		

The high turbidity and concentration of solids in the WTS samples were a challenge in terms of detecting *Giardia* spp. and *Cryptosporidium* spp. oocysts. This was also observed by Franco et al. (2012) and Silva and Sabogal-Paz (2020) [14,17], in which samples with high turbidity influenced the IMS as (oo)cysts may be adhered to solid particles, and thus not be attached by magnetic microspheres. For the WTS, aluminium was 14960 mg L^{-1} and iron was 1128 mg L^{-1}, which indicated an increase in metal concentrations resulting from the PACl as a coagulant. This process might interfere in (oo)cyst adhesion to the magnetic microspheres used in the IMS step [18].

Considering the analytical quality control with commercial suspensions, the number of protozoans inoculated in the WTS was 2224 oocysts L^{-1} and 2371 cysts L^{-1}. Oocyst and cyst recoveries were higher for the second acid dissociations, 8.7 and 13.9%, respectively. Additional dissociations would increase the final procedural costs without significantly improving recovery rates [13]. The average recovery for oocysts was 13.5 ± 7.6%, and it was 17.0 ± 3.8% for cysts, while the average viability of oocysts was 23.6 ± 2.5%, and it was 18.4

255 ± 5.6% for cysts. In this case, after applying the DC + ICN 7X method, the percentage of
256 (oo)cysts not stained with PI was higher than the initial value in the suspensions.

257 On the other hand, for the analytical quality control with EasySeed®, the highest
258 average recoveries were obtained by the first dissociations for *Cryptosporidium* spp. oocysts
259 (1.8 ± 1.5%) and for *Giardia* spp. cysts (17.8 ± 6.6%), in relation to the recovery of the
260 second for oocysts (1.5 ± 1.7%) and cysts (7.0 ± 2.1%). The coefficients of variation for
261 *Cryptosporidium* spp., both for the first and second dissociation, were considerably high
262 (84.5% and 110.5%, respectively). Considering both acid dissociations, recoveries were 3.3 ±
263 2.0% and 24.8 ± 8.0% for oocysts and cysts, respectively. For *Cryptosporidium* spp., these
264 results were less than those required for water, which is 32% according to Method 1623.1
265 [18]. *Giardia* spp. presented recovery and coefficient of variation (CV) within the
266 recommended range for water. The results for the Analytical quality assays are shown in
267 Table 2.

268

269 Table 2 - Analytical quality assays for the DC + ICN 7X followed by IMS method.

Sample	Protozoan (oocysts or cysts)	Average recovery (%)	CV (%)
Commercial suspensions [1]	*Cryptosporidium* spp.	13.5	26.4
(3 samples)	*Giardia* spp.	17.0	22.2
EasySeed® suspension [2]	*Cryptosporidium* spp.	3.3	59.7
(4 samples)	*Giardia* spp.	24.8	32.4
Method 1623.1 criteria for	*Cryptosporidium* spp.	32-100	≤ 37
water[2] (USEPA 2012)	*Giardia* spp.	8-100	≤ 39

Notes:

[1] Recovery calculated as a function of the average value of inoculated protozoans in WTS: 2224 oocysts L^{-1} and 2371 cysts L^{-1}.

[2] Recovery calculated as a function of the number of inoculated protozoans in WTS: 99.0 ± 1.6 oocysts 100 mL^{-1} and 100.0 ± 1.9 cysts 100 mL^{-1}.

[3] There is currently no standard to be followed to validate the detection protocols of protozoans in WTS.

270

271 Recoveries were smaller than those obtained by Ryu et al. [26], which used the DC
272 protocol followed by IMS, with 10 L of samples with approximately 1000 oocysts of
273 *Cryptosporidium* spp., in which recoveries were between 29.5 and 36.7% for water samples
274 with high turbidity (although less than 90 NTU). For floated residue with 380 NTU, Silva and
275 Sabogal-Paz [17] obtained lower recoveries for *C. parvum* (8.16 ± 30.24%) and achieved
276 higher values for *Giardia* spp. (32.54 ± 46.48%). Sammarro Silva and Sabogal-Paz [16]
277 generated WTS with 538 NTU and obtained *C. parvum* recoveries of 30.00 ± 8.78 % using
278 the DC + ICN 7X + IMS method.

279 The high number of organisms inoculated into the samples, approximately 2000
280 (oo)cysts L^{-1}, might influence the results of viability estimation. Another factor that may
281 explain the increase in viability was the IMS purification method, which might help recover
282 more (oo)cysts with intact cell walls than fragile or compromised ones. These observations
283 were also made by Zilberman et al. [40] in soil samples, in which the recovery was directly
284 proportional to the organisms considered viable when working with the water-ether
285 separation purification method.

286 After adding the CaO, all samples' pH achieved values higher than 12, which was
287 recommended in previous studies [33,41]. For the 3-day test, 5906 oocysts L^{-1} and 5158 L^{-1}
288 cysts were inoculated for the treatment of 27 mg of CaO/100 mL WTS samples. For oocysts,
289 the average recovery of the first acid dissociation (3.5%) was greater than the second (2.8%),
290 while the average recovery of cysts was lower in the first dissociation (2.0%) comparing to
291 the second (3.6%). However, these differences were not significant for both protozoa ($p = 0.38$). More non-stained oocysts were found in the second dissociation (2.7 ± 1.5) compared
292 to the first (1.7 ± 0.6). This fact was also observed for cysts, considering the first (1.0 ± 1.7)
293 and second dissociation (2.0 ± 2.0).

For the 5-day test, 4980 L^{-1} oocysts and 4749 L^{-1} cysts were added to the WTS samples. For oocysts, the average recovery of the first acid dissociation was higher (1.9%) than the second (0.6%), as well as in the 3-day test, but with a lower value. However, the average recovery of cysts was higher in the first dissociation (4.5%) than in the second (2.1%), different from the previous assay. These differences were statistically significant for both oocysts and cysts ($p < 0.05$). Non-stained cysts were found in the first and second acid dissociations, but only one non-stained oocyst was found in the second acid dissociation sample. The results for the WTS alkaline treatment were presented in Table 3.

There was a total recovery of 4.95% and 12.59% for oocysts and 11.11% and 13.06% for cysts, on the 3 and 5-days assays, respectively. The viability was reduced, reaching approximately 2% with a 27 mg CaO/100 mL dosage and 5 days. Even so, non-stained protozoans were still found in the samples, showing evidence of parasite resistance to the treatment. For 3 days, considering an average of 620 and 681 non-stained oocysts and cysts respectively, alkaline treatment provided 1.85 log of inactivation of *Cryptosporidium* spp. and 2.05 log of inactivation of *Giardia* spp. For 5 days, considering an average of 697 oocysts and 546 non-stained cysts, the alkaline treatment inactivated 3.00 log of *Cryptosporidium* spp. and 2.14 log *Giardia* spp..

Table 3 - Recoveries for the DC + ICN 7X method followed by IMS and percentage of parasites not stained with PI after alkaline treatment with 27 mg CaO/100 mL of WTS with contact times of 3 and 5 days, considering a multiplication factor 2.

Alkaline treatment	Protozoan	Non stained (oo)cysts (%)	CV (%)	Non stained (oo)cysts (%)	CV (%)
27 mg of CaO/100 mL WTS, 3-days, 25 °C)[1,2]	*Cryptosporidium* spp. oocysts	12.59 ± 3.31	26.30	6.19 ± 2.75	44.38
	Giardia spp. cysts	11.11 ± 3.11	27.98	5.37 ± 1.79	33.24
27 mg of CaO/100 mL	*Cryptosporidium* spp.	4.95 ± 2.26	45.57	2.22 ± 3.85	173.21

WTS, 5-days, 25 ºC)[1,3]	oocysts				
	Giardia spp. cysts	13.06 ± 7.30	55.90	2.43 ± 2.41	99.19

Notes:

[1]: One of the two centrifuge tubes was randomly chosen to apply the IMS protocol.

[2]: Estimated viability as a function of inoculated protozoan: 5906 oocysts L^{-1} and 5158 cysts L^{-1}.

[3]: Estimated viability as a function of inoculated protozoan: 4980 oocysts L^{-1} and 4749 cysts L^{-1}.

316

317 The results showed that the time interfered less in inactivating *Giardia* spp. by the
318 alkaline treatment, while the *Cryptosporidium* spp. oocysts were more resistant in the 3-day
319 test. Considering the estimated viability of (oo)cysts after the 3 and 5-day treatments, the t-
320 test was applied, considering two samples in pairs for averages, for the p-value determination,
321 adopting the confidence level of 95% ($p < 0.05$). For *Cryptosporidium* spp. oocysts, the p-
322 value was 0.19 and for *Giardia* spp. cysts, the p-value obtained was 0.14. Thus, there was no
323 significant difference between the averages and, therefore the contact time did not interfere in
324 the viability results. The microscopy images after alkaline treatment of WTS were presented
325 in Figure 2.

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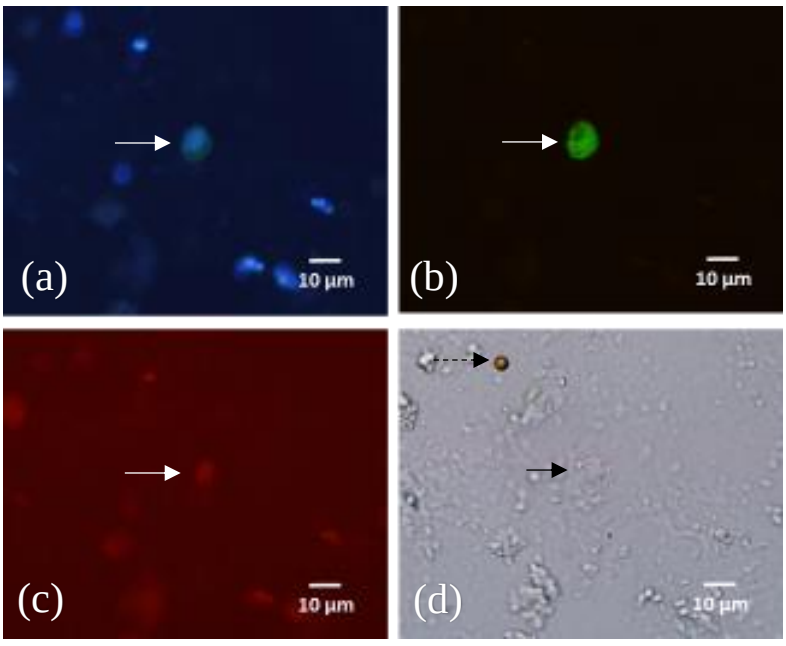

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Figure 2 - Stained *Giardia* spp. cyst after alkaline treatment (27 mg CaO/100 mL WTS of sample, 3-days at 25 °C). Parasites indicated with arrows and microspheres with dashed arrow. Images (400X): (a) DAPI; (b) FITC; (c) PI; (d) DIC.

The viability assessment through PI staining could interfere with the results obtained in the present research. For instance, Petersen and Enemark [42] discussed the protozoan underestimated viability on dry mounts, which is a limitation for this technique. Although Robertson et al. [43] had better results by adding PI in suspension compared to directly on the dried slide, this procedure may present losses throughout multiple steps and transfers. Therefore, future studies should evaluate these PI staining methodology limitations.

Although WWS sustainable recovery and disposal have been recently studied [44,45], there are also alternatives for WTS besides landfills. The potential of applying alkaline WTS for soil acidity correction could provide improvements for agricultural usages [12,46]. Other studies pointed out the WTS reuse in brick manufacturing [47], and the potential recovery of metals (e.g. aluminium and iron) from coagulants [48,49]. Therefore, this scenario reinforces the relevance of disinfecting WTS for reuse or disposal in environmental matrices (e.g. soil and water).

For protozoan analyses, approximate costs were estimated for the purchased products. Per test, the Merifluor® was US$ 17.50 per test, the Dynabeads® US$ 99, and the DAPI solution US$ 7.80. Considering Dynabeads® analysis with two slide wells (two acid dissociations), the protocol consisted of two Merifluor® and two DAPI assays, and the average price was approximately US$ 150. For tests in triplicate, the approximate cost is US$ 450. Andreoli and Sabogal-Paz [13], Giglio and Sabogal-Paz [15], Maciel and Sabogal-Paz [50] estimated the average cost of the IMS protocol of US$ 195, US$ 118, and US$ 212 per

test, respectively, values updated to April 2020 by the IGP-M/FGV. Based on this analysis's high cost, additional research should improve protozoan detection methods to reduce costs.

Approximately 150 mL WTS was produced for treating 2 L of study water. Considering a hypothetical WTPs with constant and continuous 100 L s^{-1} flow rate, the volume of WTS generated as a function of time was estimated. Thus, 7.5 L s^{-1} of WTS would be produced. The dosage of 27 mg was used per 100 mL in 3 and 5 days. Considering 20kg-bags of CaO (US$ 12.70 in local markets), 27 and 44 packages of CaO would be used, respectively for 3 and 5 days, corresponding to the total value of US$ 377 and US$ 614. These results considered only costs for the acquisition of CaO, disregarding infrastructure, equipment, personnel, and maintenance expenses.

Conclusions

Detecting protozoan in complex matrices is still a challenge for the monitoring and disposing of WTS. Although *Giardia* spp. recovery in WTS complied with the USEPA Method 1623.1 for water, the standards for WTP residues should be established. Moreover, magnetic microspheres were found attached to the protozoan in the microscope slides after IMS, indicating some limitations of this purification method. The alkaline treatment did not inactivate all parasites in the WTS, since there was non-stained protozoan even with a dosage of 27 mg of CaO/100 mL, for 5 days at 25 ºC. Oocysts were more resistant than cysts on 3 days, as cysts suffered less interference from contact time. Moreover, future research could study cheaper purification alternatives to minimize costs, which would make these procedures more accessible and feasible for monitoring protozoan in low-income places.

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Statement

Authors hereby declare previous originality check, no conflict of interest and open access to the repository of data used in this paper for scientific purposes.

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