Coagulation, Flocculation, Dissolved Air Flotation and Filtration in the Removal of *Giardia* spp. and *Cryptosporidium* spp. from Water Supply

Fernando César Andreoli and Lyda Patricia Sabogal-Paz

Department of Hydraulics and Sanitation, São Carlos School of Engineering, University of São Paulo. Brazil.

L. P. Sabogal-Paz (corresponding author), Department of Hydraulics and Sanitation, São Carlos School of Engineering, University of São Paulo, 400 Trabalhador São-carlense Avenue. Zip code: 13566-590, São Carlos, São Paulo. Brazil. Phone: +55 16 3373 9548. Fax: +55 16 33739550. e-mail: lysaboga@sc.usp.br

F. C. Andreoli, e-mail: fernandoandriolli@yahoo.com.br

This work was supported by the São Paulo Research Foundation (FAPESP-Brazil) under Grant Process 2012/50522-0; and by the National Council for Scientific and Technological Development (CNPq-Brazil) for the Master´s scholarship awarded to Fernando César Andreoli.

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Removing protozoa from a water supply using coagulation, flocculation, dissolved air flotation and filtration on a bench scale was evaluated. Flocculation in calcium carbonate with and without immunomagnetic separation was chosen to detect *Giardia* spp. cysts and *Cryptosporidium* spp. oocysts in the studied samples. Analytical quality of assays of the detection protocol of target organisms was performed and the results were compared to the criteria established in Method 1623.1 from the US Environmental Protection Agency. The results indicated that dissolved air flotation removed between 1.31 log and 1.79 log of cysts and between 1.08 log and 1.42 log of oocysts. The performance was lower in filtration, with the removal of 1.07 log to 1.44 log for cysts and 0.82 log to 0.98 log for oocysts. The coagulation, flocculation, dissolved air flotation and filtration steps removed more than 2.2 log of cysts and oocysts from the water studied. However, protozoa were detected in the filtered water, even with turbidity values of 0.2 NTU. The recovery of the detection method met the international criteria and was higher when there was no immunomagnetic separation. Including the third acid dissociation in the immunomagnetic separation was critical to improve the performance of the protocol tested. However, there was an increase in the technical and analytical complexity and costs. It was also observed that the efficiency of the treatment was linked to the performance of the selected method of detecting protozoa.

Keywords: bench scale, drinking water, immunomagnetic separation, polyaluminium chloride, protozoa

Introduction

Water-borne diseases associated with *Giardia* spp. and *Cryptosporidium* spp. protozoa have been recorded around the world [1,2]. According to Efstratiou et al. [3], 82% of the cases documented between 2011 and 2016 presented different transmission routes including contamination of water sources and problems in water supply systems. When

water treatment does not remove these parasites, the population supplied with the water becomes an easy target for diseases that affect their quality and routine of life. An example of this was an outbreak of cryptosporidiosis recorded in northern Sweden in 2011, which affected approximately 6,000 people and forced the population to boil their water for months [4].

Protozoa are identified as etiologic agents in waterborne disease outbreaks in less-developed countries. Cryptosporidiosis in Africa is a major cause of diarrhea, especially in children and HIV patients [5]. In Latin America countries, methods to detect protozoa in water samples have not been well-defined, leaving the population vulnerable to waterborne infections. Brazil and Colombia have implemented laws in their surveillance systems to detect protozoa, however it is important to establish effective and suitable microbiological risk diagnosis according to the economic strength and particular needs of each country [6].

Ingesting small amounts of *Giardia* spp. cysts or *Cryptosporidium* spp. oocysts is sufficient to cause bouts of diarrhea and in immunocompromised persons the clinical condition can cause death. These protozoa need a host to complete their life cycles. However, recent research has indicated that the genus *Cryptosporidium* may be able to reproduce in aquatic biofilms, thus leading to new implications in the area of sanitation [7, 8]. Mahmoudi et al. [9] analyzed the situation of cryptosporidiosis in Asia and they observed the highest prevalence of the parasite where there was greater contact between humans and animals and in situations where there was precarious sanitation.

The presence of pathogenic protozoa in water supply systems poses a health risk and a challenge to governments because detecting, removing and inactivating these parasites incur high costs, as well as technical and analytical complexity [10].

Results obtained by numerous methods of protozoa detection show the need of standardize concentration and purification procedures in different laboratories worldwide to determine the most economical and efficient method to be selected for public health surveillance [11].

The US Environmental Protection Agency (USEPA) Method 1623.1 [12] is the most widespread protocol for detecting protozoa in drinking water. Nonetheless, this method must be adapted to the reality of developing countries, aiming to estimate (using available resources) the risk attributed to *Giardia* and *Cryptosporidium* protozoa. Evidently, changes to Method 1623.1 are predicted, according to the USEPA [12], when the performance of the alternative protocol is greater and the costs and processing time of the samples are reduced.

Maciel and Sabogal-Paz [10] evaluated the removal of *Giardia* spp. cysts and *Cryptosporidium* spp. oocysts from water with high turbidity in jar tests using polyaluminium chloride (PACl) as a coagulant. Filtration using mixed cellulose ester membranes, followed, or not, by purification through immunomagnetic separation (IMS) was used for detecting protozoans. The authors found low oocyst recoveries (without using IMS, retrievals of 80% of cysts and 5% of oocysts were obtained, whereas by using IMS, recoveries of 31.5% of cysts and 5.75% of oocysts were reached). According to the authors, other simplified methods need to be tested, aiming to detect oocysts in environmental samples in Latin America countries, such as flocculation-sedimentation methods.

Concerning the detection of protozoa, the filtration technique has been the most popular method to isolate protozoan parasites from water, however it has limitations in sample volumes due to filter clogging. An alternative to monitoring protozoa in water is

flocculation-sedimentation, based on the difference in the relative weight of the parasite and the impurities [13].

Various flocculation-sedimentation methods are available, such as Calcium Carbonate Flocculation (CCF), developed by Vesey et al. [14], which has been further developed in environmental samples with and without IMS [15,16].

CCF has been a cost effective alternative to the filtration technique, particularly in water samples with high turbidity levels [16]. Other flocculation-sedimentation methods have also used ferric sulphate and aluminium sulphate for the purification of *C. parvum* oocysts from tap water [17].

CCF is promising in terms of samples with high turbidity, such as residue generated by water treatment and it has a reduced cost compared to the Filtra-Max® filtration technique. In this context, evaluating this protocol in environmental matrices may be relevant in developing countries. According to Campbell et al. [18], CCF reduced approximately 30% of the viability of *C. parvum* oocysts when two fluorogenic dyes were used. The comparison was made when evaluating two concentration methods (direct centrifugation and CCF). Evidently, results involving CCF together with viability or animal infectivity testing should be interpreted with caution.

Karanis and Kimura [17] observed that *in vitro* excystation experiments, ferric sulphate flocculation does not markedly reduce the viability of *C. parvum* oocysts and this method had the highest recovery (61.5%) when compared with calcium carbonate (38.8%) and aluminium sulphate (58.1%). Obviously, the performance of each protozoa detection method varies depending on the water sample.

Regarding water treatment, significant advances have been recorded in recent years. However, there are still reports of disease transmission related to water supply, including developed countries [19,3]. The challenge of removing these protozoa begins

when evaluating the reduced size (cysts of 10-12 μm and oocysts of 4-6 μm) which favor the direct passage through filters in Water Treatment Plants (WTPs) and their low density with approximately 1070 kg/m^3 [20], which makes it difficult to remove them in conventional WTPs. In addition, these protozoa can survive for long periods in the environment and are resistant to commonly used disinfectants [21,22].

Water clarification with Dissolved Air Flotation (DAF) has shown to be more efficient than decantation when removing *Giardia* cysts and *Cryptosporidium* oocysts, i.e. when flocs formed after coagulation-flocculation are light, resulting from water treatment with low mineral turbidity [23,24,20].

DAF has been used to treat drinking water in several countries, such as Finland, the United Kingdom, Australia, Korea, Malaysia, Hong Kong, South Africa, Scandinavia, the United States, Canada and Brazil [25].

In WTPs with DAF, raw water is coagulated and rapid mixing can be hydraulic or mechanized, depending on the flow to be treated, the variation of the raw water quality and the available operating and maintenance conditions. Afterwards the coagulated water is subjected to slow mixing, usually mechanized until the flocs reach a sufficient size so that they float by using pressurized recirculation water. Then, the clarified water is filtered and disinfected [26].

Edzwald and Harrhoff [20] cited various reasons for choosing DAF in water clarification rather than decantation, such as the efficiency in removal algae, pathogens, true color, taste and odor; the possibility of generating more concentrated sludge; and the benefit of occupying less area in the plant. Nevertheless, in relation to the costs, Kawamura [27] indicated that the equipment used both for the air saturation system and for the sludge removal system significantly increases installation, maintenance and

operation costs. Thus, a WTP with DAF tends to incur more expenses when compared to a similar WTP involving decantation.

DAF treatment can be simulated using bench scale testing, therefore some design parameters can be obtained quickly and economically. The DAF bench-scale jar test unit can be used for this purpose, consisting of a pressurized chamber, jars, dosing and collection sets and an agitator motor. In some cases, in the DAF jar test it is possible to install laboratory filters to simulate the complete treatment, from coagulation to filtration. Among the possible variables to be studied on a bench scale, Edzwald and Harrhoff [20] recommended a flocculation time from 5 to 20 min, a flotation time of 10 min, a saturation pressure of 400 to 600 kPa and a recirculation rate from 6 to 12%.

In this context, the article evaluated the removal of *Giardia* spp. cysts and *Cryptosporidium* spp. oocysts throughout the treatment concerning coagulation, flocculation, DAF and filtration on a bench scale. The performance of the CCF method with and without IMS to detect protozoa in environmental samples (raw water, clarified water, filtered water, floated residue and filter backwash water) was also studied, considering the use of two and three dissociations acids in IMS. The estimated costs of the assays were calculated considering only the high cost reagents. The Student's t-test for a 95% confidence level was used to verify if there were significant statistical differences between the tested methods (CCF with or without IMS).

Methods

The study was divided into six stages. Step 1 consisted of preparing the water studied by adding 0.09 gL^{-1} kaolinite and 0.01 gL^{-1} of humic acid to well water (without cysts or oocysts), which simulates the color and turbidity conditions of raw water commonly used in treatments with DAF. This water was designed to eliminate possible interferences inherent to the natural samples as the purpose was to weigh the

performance of the selected detection protocol (CCF with and without IMS) and the distribution of the cysts and oocysts throughout the treatment.

DAF jar test assays were carried out in Step 2 to optimize the design parameters (coagulant dosage, coagulation pH, mixing conditions, flotation time and recirculation rate). The PACl was used with Al_2O_3 content of 17.66%. Laboratory filters filled with sand (grains between 0.30 mm and 0.59 mm and effective size of 0.42 mm) were coupled to the DAF jar test, which operated at a rate of 100 $m^3.m^{-2}d^{-1}$. The efficiency of the treatment was estimated as a function of the color and turbidity of the filtered water. When optimizing the design parameters, new DAF jar tests were run to collect and characterize the samples (clarified water, filtered water, floated residue and filter backwash water). The physico-chemical and microbiological analyses carried out followed the procedures described in APHA et al. [28].

Step 3 evaluated the performance of the CCF method with and without IMS to detect protozoa. Analytical quality assays were performed in the water studied by inoculating a number of known organisms (EasySeed® and AccuSpike®) followed by adopting the method to evaluate recovery. The results were compared with the criteria from the USEPA Method 1623.1 [12], considering the data obtained from four assays, under equal conditions, plus a blank one.

The CCF first involved the formation of flocs by adding sodium bicarbonate (1M, 10 $mL.L^{-1}$ sample) and calcium chloride (1M, 10 $mL.L^{-1}$ sample) and pH adjustment to 10 with 5M sodium hydroxide. The mixture remained at room temperature overnight. After the time described, the supernatant was discarded, leaving 100 mL of the sample. It was then shaken for 10 min and sulfamic acid was added (10%, 20 $mL.L^{-1}$ sample) and it was stirred for a further 5 min. The sample was transferred to a 50 mL Falcon® tube and centrifuged at 1500xg for 20min. The pH of

the centrifuged sample was corrected until reaching neutrality by adding aliquots of Phosphate Buffered Saline (PBS). A pellet with less than 0.5 mL was obtained and the concentrated sample was sent for purification via IMS (CCF with IMS) or for direct reading under the microscope (CCF without IMS).

When direct reading in the microscope was carried out, three aliquots of 50 µL were taken from the centrifuged sample mentioned above. Reagents from the Merifluor Meridian® kit and Fluoroshield with DAPI-Sigma-Aldrich® solution were added (according to the manufacturers´ recommendations) to each well containing the samples. The parasites were counted and enumerated under a microscope (200X to 800X). Visualization was performed in Fluorescein Isothiocyanate (FITC) in conjunction with 4',6-diamidino-2-phenylindole (DAPI) and Differential Interference Contrast (DIC). The recovery was calculated, according to Equation 1:

$$R = \left[\frac{(P_1+P_2+P_3).CF}{NP}\right] x100\% \quad (1)$$

Where R: recovery of CCF without IMS (%); P_1, P_2 and P_3: cysts or oocysts visualized in the three wells of the immunofluorescence slide; NP: number of inoculated protozoa; CF: correction factor of 6.7 which was calculated by dividing the volume of the concentrated sample (1.0 mL) by the volume examined in the wells (0.15 mL).

The IMS was carried out using the Dynabead® kit (life Technologies™), according to the manufacturer's instructions, considering two and three acid dissociations. The buffer solutions and *Anti-Giardia* and *Anti-Cryptosporidium* microspheres present in the kit were added to the sample and mixed in a rotating mixer for 1 hour at 18 rpm. Thereafter, the sample was placed on two magnetic particle concentrators, which separated the microspheres/protozoa set from the rest of the sample. Successive dissociations were carried out by applying 50 µL of hydrochloric

acid 0.1 N for 10 min. At the end of each dissociation, the sample was placed back into the particle concentrator and the liquid was removed for the microscope slides and 5 μL of sodium hydroxide 1.0 N were added for neutralization.

At the end of the IMS, the microscope slide was prepared. The reagents from the Merifluor kit and the DAPI solution were added to each well containing the samples. The parasites were counted and enumerated under a microscope. The visualization was performed in FITC, DAPI and DIC. Finally, the recovery percentage was calculated, according to Equation 2.

$$R = \left[\frac{(C_1+C_2+C_3)}{NP}\right] \times 100\% \quad (2)$$

Where R: recovery of CCF with IMS (%); C_1: counting of cysts or oocysts in the first acid dissociation; C_2: counting of cysts or oocysts in the second acid dissociation; C_3: counting of cysts or oocysts in the third acid dissociation; and NP: number of inoculated protozoa.

Step 4 consisted of counting the protozoa in the suspensions and subsequent inoculum of the parasites in the samples. Purified suspensions of *Giardia* spp. cysts and *Cryptosporidium* spp. oocysts were used by inoculating a known number of target organisms into the vessels of the DAF jar test (between 10^2 and 10^3 cysts.L^{-1} and oocysts.L^{-1}).

Purified suspensions of *Giardia* spp. cysts, aged 4 to 6 months, were acquired from the Protozoology Laboratory at the University of Campinas, Brazil. The cysts were isolated by sucrose density gradient centrifugation of human feces, stored in ultrapure water with antibiotics and enumerated by an immunofluorescence well assay. *Cryptosporidium* spp. oocysts, aged 4 to 6 months, were purchased from Waterborne, USA. Oocysts were purified from calves' feces by sucrose and Percoll density gradient

centrifugation, after initial extraction of feces with diethyl ether. Enumeration was performed by Neubauer hemacytometer using the red blood cell method.

The inoculum volume to obtain the desired concentration of protozoa in the sample was determined by the arithmetic mean of cysts and oocysts counted in three identical aliquots taken after homogenization of the suspensions. The counting was done by microscope in FITC, DAPI and DIC. At the end of the procedure, the inoculum was added to the samples in the DAF jar test and treatability testing was carried out using the parameters optimized in Step 2.

In Step 4, the removal of cysts and oocysts throughout the treatment was evaluated considering three phases. In Phase 1, coagulation, flocculation and DAF were used together and, in this case, the inoculum was directly added to the water studied. Phase 2 evaluated the filtration performance, therefore the inoculation was carried out in the clarified water (collected in Step 2). Phase 3 estimated the coagulation, flocculation, DAF and filtration performance to remove the parasites. Thus, the inoculum was carried out in the water studied. The tests, in each of the phases, were performed in triplicate using the DAF jar test.

Step 5 comprised a statistical hypothesis test, using a Student's t-test and a 95% confidence level to evaluate whether there were statistical differences between protocol recoveries (CCF with and without IMS).

Finally, in Step 6, the costs of the main reagents used (Merifluor® kit, Easyseed®, AccuSpike® and Dynadeads® kit) were analyzed for Brazil in order to economically compare the protocol tested. In this case, the average and standard deviation of the quotations obtained were calculated and the prices were updated for April, 2016 using the General Market Price Index (IGP-M in Portuguese) from the *Fundação Getúlio Vargas*.

Results and Discussion

The optimal treatment conditions are shown in Table 1 and were obtained without needing to adjust the pH of the water studied. These values are close to those recommended by Edzwald and Harrhoff [20] in bench tests.

[Table 1 near here]

The parasite recovery in the analytical quality assay for the CCF method with and without IMS is shown in Table 2. The results obtained met the acceptance criteria of the USEPA Method 1623.1 [12], with greater recovery when the IMS was not performed, a phenomenon also reported by Maciel and Sabogal-Paz [10], using a different protozoan detection protocol (filtration through mixed cellulose ester membranes with and without IMS).

[Table 2 near here]

The recovery of oocysts was greater, regardless of the protocol tested, according to Table 2. This result was contrary to that obtained by Maciel and Sabogal-Paz [10], when another method of protozoan detection was used. The third acid dissociation in IMS increased the recovery of cysts by 8.0% and oocysts by 14.2%. The results were similar to those obtained by Chang et al. [29] as they evaluated the influence of the number of acid dissociations on the IMS in relation to the recovery of *C. parvum* using the deionized water matrix. The authors observed that between the second and third acid dissociations, the recovery increased by approximately 19% and also found that from the third dissociation, the accumulated number of recovered parasites remained constant.

According to the results of the hypothesis test (Student's t-test with 95% confidence level), the CCF method without IMS had a significantly higher recovery than the IMS method and two acid dissociations, for both protozoa tested. Including a

third dissociation, the method without IMS remains statistically different only for *Cryptosporidium* spp.

The purification phase by IMS allowed a better visualization of the cysts and oocysts because the debris was reduced. However, adopting the method, with various stages of handling the sample, can generate a loss of target organisms. Thus, by excluding the IMS, the number of delicate procedures is reduced, and the chances of further recovery are likely to increase.

The costs of the analytical quality assay of the CCF method with and without IMS are shown in Table 3, considering the prices quoted in Brazil. Including the third acid dissociation in IMS increased, on average, the price of the test by 11.7% and, in addition, it was observed that CCF without IMS had a cost reduction from 25.8% to 34.5%.

[Table 3 near here]

According to USEPA [12], the analytical quality assay should be performed on every 20 environmental samples processed. Therefore, the annual costs associated with monitoring these parasites in developing countries are restrictive in order to assess the risk of infection attributed. Evidently, these costs are substantially higher compared to those required for monitoring parameters such as turbidity and *Escherichia coli*.

Table 4 shows the distribution and removal of protozoa throughout the treatment involving coagulation, flocculation and DAF.

[Table 4 near here]

In Phase 1, the removal of protozoa ranged from 1.31 log to 1.79 log for cysts and from 1.08 log to 1.42 log for oocysts. This performance was lower than that reported by Plummer et al. [23]. According to the authors, more than 2 log removal of *C. parvum* oocysts were reached on a bench scale when coagulation, flocculation and

DAF were used. The chosen method to detect protozoa consisted of centrifugation and flotation in sucrose. In this study, ferric chloride was the coagulant used and 3 to 4 x 10^5 oocysts.L^{-1} were inoculated into the water studied.

On the other hand, Edzwald et al. [24] stated that DAF clarification was more efficient than decanting, with mean removals of *Giardia* cysts and *Cryptosporidium* oocysts between 2.4 log ± 0.3 and 2.1 log ± 0.3, respectively. The protozoan detection protocol comprised centrifugation and visualization under epifluorescence microscope. Evidently, these differences among the cited results are related to the characteristics of the matrix, the number of organisms inoculated and the detection method selected.

The largest removal of cysts and oocysts occurred when CCF with two acid dissociations was used - the protocol with the highest inherent losses (79.9%±2.2 for cysts and 21.8%±14.6 for oocysts), according to Table 4. Unfortunately, these losses are counted as organisms removed in the efficiency calculation. Therefore, it can be observed that the effectiveness of the treatment is linked to the performance of the selected method to detect protozoa.

Removal of protozoa by filtration is shown in Table 5 and a greater presence of oocysts in the filtered water was detected, regardless of the selected detection protocol (CCF with and without IMS). This lower removal of *Cryptosporidium* spp. may be related to the capacity of the oocysts to squeeze and fold - conditions necessary to cross the pores of the filter media, a phenomenon described by Li et al. [30].

[Table 5 near here]

The number of protozoa inoculated in the clarified water (452 to 869 cysts.L^{-1} and 547 to 646 oocysts.L^{-1}) clearly influenced the high number of protozoa present in the filtered water (11 to 93 cysts and 46 to 133 oocysts). This phenomenon was also reported by Swertfeger et al. [31], when evaluating the performance of various filter

media in the removal of protozoa. Thus, it is necessary to use the other water treatment processes before filtration besides disinfection.

Occurrence of protozoa in the filter backwash water shows the importance of appropriate treatment (Table 5). Karanis et al. [32] also detected *Giardia* cysts and *Cryptosporidium* oocysts in this matrix and they warned of the hazard of recycling this residue into WTPs.

The removal of protozoa, in Phase 2, was in the range of 1.07 log to 1.44 log for cysts and from 0.82 log to 0.98 log for oocysts and, clearly, this performance was lower than that observed in DAF (Phase 1). Studies conducted by Swertfeger et al. [31] achieved removals of more than 2.5 log of cysts and oocysts in pilot filters operated. However, in the aforementioned study, approximately 10^5 to 10^6 organisms were inoculated into the filter affluent (clarified water obtained after coagulation, flocculation and decantation) and the protozoan detection method used consisted of filtration, centrifugation and visualization under epifluorescence microscope. Again, the characteristics of the matrix, the number of inoculated organisms and the detection method generated differences in results between the research.

Regarding the inherent losses, CCF with IMS (two or three acid dissociations) presented the highest losses for cysts and oocysts; it was higher for the first parasite. Once more, the CCF with IMS protocol and two acid dissociations obtained the greatest efficiencies (1.44 log for cysts and 0.98 log for oocysts); nevertheless, linked with higher inherent losses (81.3% ± 4.4 for *Giardia* spp. and 29.9% ± 12.9 for *Cryptosporidium* spp.).

Table 6 shows the distribution and removal of protozoa throughout the treatment involving coagulation, flocculation, DAF and filtration. The inherent losses were higher

in CCF with IMS (two or three acid dissociations). The highest was for cysts (67.4% ± 7.4 to 90% ± 3.6).

[Table 6 near here]

Removal of protozoa throughout the treatment, regardless of the detection method used (CCF with and without IMS), was higher than 2.2 log. This performance was lower than that reported by Edzwald et al. [24] when DAF was studied followed by filtration, on a pilot scale. These authors obtained approximately 5 log removals for *Giardia* and *Cryptosporidium* when working with low turbidity water, aluminum sulphate and cationic polymer as coagulants, and centrifugation and visualization in epifluorescence microscope as a method to detect parasites. Once again, the characteristics of the matrix, the number of inoculated organisms and the detection method generated the differences reported in the cited results.

Cysts and oocysts were detected in filtered water, even with turbidity of 0.2 NTU, regardless of the detection method used (CCF with or without IMS). Therefore, treatment by coagulation, flocculation, DAF and filtration does not completely remove the protozoa studied. Thus, the importance of disinfecting the filtered water in order to reduce the microbiological risk present in drinking water is essential.

The value of a single assay to detect protozoa in any matrix, considering only the most expensive reagents, is shown in Table 7. The CCF with IMS and three acid dissociations increased expenditures by 17% when CCF was compared with two dissociations. CCF without IMS had a lower cost, between 39% and 50%. According to Maciel and Sabogal-Paz [10], the cost of reagents (Merifluor and Dynabeads kits) was estimated at US$198 and US$190 for assays with and without IMS, respectively in June 2015. Thus, the use of IMS increased the costs by almost 4% when another method of protozoan detection was used (filtration through membranes with and without IMS). In

any case, the values described were significantly higher compared to the costs of other routine tests required to monitor a water supply system.

[Table 7 near here]

Conclusions

Coagulation, flocculation and DAF removed between 1.31 log and 1.79 log cysts and between 1.08 log and 1.42 log oocysts, and the performance was lower when weighting only the filtration, with removal of 1.07 log to 1.44 log for cysts and 0.82 log to 0.98 log for oocysts. The coagulation, flocculation, dissolved air flotation and filtration steps removed more than 2.2 log cysts and oocysts from the water studied. However, protozoa were detected in the filtered water, even with turbidity values of 0.2 NTU.

The method used (CCF with and without IMS) met the analytical quality criteria established by Method 1623.1 and the smallest recoveries were recorded for *Giardia* spp. cysts. The statistical hypothesis test indicated that the CCF method without IMS had a significantly higher recovery than the IMS method and two acid dissociations for both protozoa tested. Including a third dissociation, the method without IMS remained statistically different only for *Cryptosporidium* spp..

The efficiency of the treatment is linked to the performance of the selected method to detect protozoa, an aspect that should be taken into consideration by the researchers when evaluating tested or implemented treatment techniques.

The cost of a single analytical quality assay of the tested protocol was over US$ 1,105 considering only the higher cost reagents. In addition, the expense for a single protozoan test for any environmental matrix was over US$ 118. These amounts are significantly higher compared to the costs of other routine tests required to monitor a water supply system. These prices are a deterrent to many sanitation companies in

developing countries, and this aspect certainly restricts establishing surveillance systems.

The results and experiences of this study indicated that more research is needed to properly select protozoa detection methods in environmental samples according to the technical and economic capacity of developing countries. Reducing the complexity of the assay and minimizing costs, not needing to purchase imported products, are essential to assess the microbiological risk in water supply in these countries.

Acknowledgements

The authors are grateful to the São Paulo Research Foundation (FAPESP) for the research support (Process 2012/50522-0) and the National Council for Scientific and Technological Development (CNPq-Brazil) for the Master´s scholarship awarded to Fernando César Andreoli.

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Table 1. Parameters obtained in the DAF jar test with coupled laboratory filters

Parameter	Values
PACl dosage ($mg.L^{-1}$)	25
Al^{3+} concentration ($mg.L^{-1}$)	2.19
pH coagulation	6.64
Mean velocity gradient in rapid mixing (s^{-1})	700 (350 rpm)
Rapid mixing time (s)	10
Mean velocity gradient for flocculation (s^{-1})	60 (65 rpm)
Flocculation time (min)	4
Flotation time (min)	10
Recirculation rate (%)	5
Filtration rate ($m^3.m^{-2}.d^{-1}$)	100
Saturation pressure	500 kPa

Characteristics of studied water: turbidity = 54.8 NTU; real color = 86.8 HU; pH = 6.64; zeta potential = 25.2 mV; electrical conductivity = 60.7 $\mu S.cm^{-1}$; absorbance (λ254nm) = 0.259; total alkalinity = 51 mg $CaCO_3.L^{-1}$; total coliforms = 1 $NMP.100mL^{-1}$; *Escherichia coli* =1 $NMP.100mL^{-1}$; TOC = 2.2 $mg.L^{-1}$; total aluminum = 0.69 $mg.L^{-1}$; total iron = 0.26 $mg.L^{-1}$; and total manganese = 0.006 $mg.L^{-1}$.

Characteristics of clarified water: turbidity = 3.64 NTU; pH = 6.69; zeta potential = -0.611 mV; electrical conductivity = 56.67 $\mu S.cm^{-1}$; absorbance (λ254nm) = 0.017; total alkalinity = 35 mg $CaCO_3.L^{-1}$; total aluminum = 0.83 $mg.L^{-1}$; total iron = 0.06 $mg.L^{-1}$; and total manganese = 0.006 $mg.L^{-1}$.

Characteristics of filtered water: turbidity = 0.2 NTU; real color = 6.8 HU; pH = 6.8; zeta potential = -0,22 mV; electrical conductivity = 53.2 $\mu S.cm^{-1}$; absorbance (λ254nm) = 0.016; total alkalinity = 32 mg $CaCO_3.L^{-1}$; total aluminum= 0.21 $mg.L^{-1}$; total iron = 0.06 $mg.L^{-1}$; and total manganese = 0.019 $mg.L^{-1}$.

Characteristics of floated residue: turbidity = 459 NTU; pH = 6.73; zeta potential = -0.169 mV; electrical conductivity = 41.95 $\mu S.cm^{-1}$; absorbance (λ254nm) = 0.028; total alkalinity = 36 mg $CaCO_3.L^{-1}$; total solids = 710 $mg.L^{-1}$; total suspended solids = 480 $mg.L^{-1}$; TOC = 11.4 $mg.L^{-1}$; DQO = 105.8 $mg.L^{-1}$; total aluminum = 0,83 $mg.L^{-1}$; total iron = 0.063 $mg.L^{-1}$; and total manganese = 0.006 $mg.L^{-1}$.

Characteristics of filter backwash water: turbidity = 3.56 NTU; pH = 6.42; zeta potential = 0.454 mV; electrical conductivity = 15.47 $\mu S.cm^{-1}$; absorbance (λ254nm) = 0.012; total alkalinity= 10 mg $CaCO_3.L^{-1}$; total solids = 120 $mg.L^{-1}$; total suspended solids = 10 $mg.L^{-1}$; TOC = 0.048 $mg.L^{-1}$; total aluminum = 1.17 $mg.L^{-1}$; total iron = 0.1 $mg.L^{-1}$; and total manganese = 0.014 $mg.L^{-1}$.

Table 2. Recovery of the analytical quality assay of the CCF method with and without IMS for two and three acid dissociations (matrix evaluated = water studied)

Tested protocols	*Giardia* spp. Cysts		*Cryptosporidium* spp. Oocysts	
	Recovery (%)	CV (%)	Recovery (%)	CV (%)
CCF without IMS[1]	31.5	20.2	70.3	12.3
CCF with IMS (2D)[2]	16.7	2.9	32.3	5
CCF with IMS (3D)[2]	24.7	4.1	46.5	6.1
Criteria of USEPA (2012)	8 – 100	≤ 97	32 - 100	≤ 46

[1] AccuSpike® test; [2] EasySeed® test; 2D: test with two dissociations; 3D: test with three dissociations; CV: coefficient of variation; CCF: calcium carbonate flocculation; and IMS: immunomagnetic separation. Four tests plus blank were performed under equal conditions.

Table 3. Cost of the analytical quality assay of the CCF method with and without IMS (values refer to Brazil, updated on April 1, 2016 by the IGP-M index).

Method	Cost in dollars - US$ (average ± standard deviation) Number of quotations = 5
CCF without IMS[1]	1105,9 ± 138,0
CCF with IMS (2D)[2]	1490,6 ± 370,3
CCF with IMS (3D)[3]	1687,4 ± 389,6

2D: two acid dissociations; 3D: three acidic dissociations; CCF: calcium carbonate flocculation; and IMS: immunomagnetic separation; [1]: cost calculated for only 4 units of AccuSkike® and 15 units of Merifluor® kit; [2]: cost calculated for only 4 units of Easyseed®, 10 units of the Merifluor® kit and 5 units of the Dynadeads® kit; [3]: calculated cost considering only 4 units of Easyseed®, 15 units of the Merifluor® kit and 5 units of the Dynadeads® kit. Exchange rate: 1 US$ = $3.58

Table 4. Distribution and removal of protozoa when coagulation, flocculation and DAF were used in the treatment (triplicate test results) - Phase 1

Evaluated matrix	*Giardia spp*. cysts accounted for in the matrices									*Cryptosporidium* spp. oocysts accounted for in the matrices								
	CCF without IMS			CCF (2D) with IMS			CCF (3D) with IMS			CCF without IMS			CCF (2D) with IMS			CCF (3D) with IMS		
	Tests									Tests								
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Studied water (SW)*	1055	1055	1055	1465	1465	1465	1465	1465	1465	1076	1076	1076	1370	1370	1370	1370	1370	1370
Clarified water (CW)	33	67	60	23	27	22	32	46	37	73	107	93	82	61	28	152	73	40
Floated residue (FR)	453	767	727	298	277	237	467	471	413	913	1027	947	848	1152	1386	977	1271	1514
Inherent losses (IL) = SW-CW-FR	568	222	268	1144	1161	1206	966	948	1015	89	NAL	36	440	157	NAL	241	26	NAL
IL (%)	53.9	21.0	25.4	78.1	79.2	82.3	65.9	64.7	69.3	8.3	NAL	3.3	32.1	11.5	NAL	17.6	1.9	NAL
-Average (%)	33.4			79.9			66.6			5.8			21.8			9.7		
-Standard deviation (%)	17.8			2.2			2.4			3.5			14.6			11.1		
Log removals																		
In flotation (SW-CW)/SW	1.50	1.20	1.25	1.80	1.73	1.82	1.66	1.50	1.60	1.17	1.00	1.06	1.22	1.35	1.69	0.95	1.27	1.53
-Average	1.31			1.79			1.59			1.08			1.42			1.25		
-Standard deviation	0.2			0.0			0.1			0.1			0.2			0.3		

2D: two acid dissociations; 3D: three acid dissociations; CCF: calcium carbonate flocculation; IMS: immunomagnetic separation; * mean value of cysts and oocysts obtained in triplicate tests; NAL: no apparent losses; and IL: inherent losses to the protocol tested.

Table 5. Distribution and removal of protozoa when filtration was used in the treatment (triplicate test results) - Phase 2

Evaluated matrix	*Giardia spp*. cysts accounted for in the matrices									*Cryptosporidium* spp. oocysts accounted for in the matrices								
	CCF without IMS			CCF with IMS (2D)			CCF with IMS (3D)			CCF without IMS			CCF with IMS (2D)			CCF with IMS (3D)		
	Tests									Tests								
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Clarified water (CW)*	869	869	869	452	452	452	452	452	452	547	547	547	646	646	646	646	646	646
Filter backwash water (FBW)	507	587	567	72	59	46	141	140	118	547	440	380	334	378	422	431	483	515
Filtered water (FW)	67	93	67	25	16	11	49	34	17	73	80	100	54	46	125	58	47	133
Inherent losses (IL) = CW-FBW-FW	296	189	236	355	377	395	262	278	317	NAL	27	67	258	222	99	157	116	NAL
IL (%)	34.0	21.7	27.1	78.5	83.4	87.4	58.0	61.5	70.1	NAL	4.9	12.2	39.9	34.4	15.3	24.3	18.0	NAL
-Average (%)	27.6			83.1			63.2			8.6			29.9			21.1		
-Standard deviation (%)	6.2			4.4			6.3			5.2			12.9			4.5		
Log removals																		
In filtration (CW-FW)/CW	1.12	0.97	1.12	1.26	1.45	1.61	0.96	1.12	1.42	0.87	0.83	0.74	1.08	1.15	0.71	1.05	1.14	0.69
- Average	1.07			1.44			1.17			0.82			0.98			0.96		
- Standard deviation	0.1			0.2			0.2			0.1			0.2			0.2		

2D: two acid disassociations; 3D: three acid disassociations; CCF: Flocculation in calcium carbonate; IMS: immunomagnetic separation; * Mean value of inoculated cysts and oocysts; IL: inherent losses; NAL: no apparent losses

Table 6. Distribution and removal of protozoa when coagulation, flocculation and DAF were used in the treatment (triplicate test results) - Phase 3

Evaluated matrix	*Giardia spp*. cysts accounted for in the matrices									*Cryptosporidium* spp. oocysts accounted for in the matrices								
	CCF without IMS			CCF with IMS (2D)			CCF with IMS (3D)			CCF without IMS			CCF with IMS (2D)			CCF with IMS (3D)		
	Tests									Tests								
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Water studied (WS)*	1622	1622	1622	476	476	476	476	476	476	1578	1578	1578	1743	1743	1743	1743	1743	1743
Floated residue (FR)	793	787	853	52	56	24	166	170	111	1733	913	1287	1530	1671	819	1689	1715	861
Filtered backwash water (FBW)	67	80	47	3	4	2	5	9	2	107	133	73	83	102	74	101	104	88
Filtered water (FW)	0	13	7	0	0	2	0	1	2	7	20	7	2	1	4	8	6	4
Inherent loss (IL) = WS-FR-FW-FBW	762	742	715	421	416	448	305	296	361	NAL	511	211	128	NAL	846	NAL	NAL	790
IL (%)	47.0	45.7	44.1	88.4	87.4	94.1	64.1	62.2	75.8	NAL	32.4	13.4	7.3	NAL	48.5	NAL	NAL	45.3
-Average		45.6			90.0			67.4			22.9			27.9			45.3	
-Standard deviation		1.4			3.6			7.4			13.4			29.1			NSD	
Log removals																		
Complete treatment (WS-FW)/WS	TR	2.09	2.39	TR	TR	2.38	TR	2.68	2.38	2.37	1.90	2.37	2.94	3.24	2.64	2.34	2.46	2.64
-Average		2.24			2.38			2.53			2.22			2.94			2.48	
-Standard deviation		0.2			NSD			0.2			0.28			0.3			0.2	

2D: two acid dissociations; 3D: three acid dissociations; CCF: calcium carbonate flocculation; and IMS: immunomagnetic separation; TR: total removal; NAL: no apparent losses; NSD: no standard deviation; * Mean value of cysts and oocysts obtained in triplicate tests; IL: inherent losses

Table 7: Cost of a single protozoal detection test (CCF with and without IMS) in any environmental matrix on April 1, 2016 (data from Brazil updated by the IGP-M index)

Method	Cost for the processing of a single sample in dollars - US $ (average ± standard deviation). Number of quotations = 5
CCF without IMS (2D)[1]	118,0 ± 26,6
CCF with IMS (2D)[2]	195,0 ± 78,3
CCF with IMS (3D)[3]	234,3 ± 82,0

2D: two acid dissociations; 3D: three acid dissociations; CCF: calcium carbonate flocculation; IMS: immunomagnetic separation; [1]: cost calculated or only 3 units of the Merifluor® kit; [2]: cost calculated for only 2 units of the Merifluor® kit and 1 unit of the Dynadeads® kit; [3]: calculated cost considering only 3 units of the Merifluor® kit and 1 unit of the Dynadeads® kit. Exchange rate: 1 US $ = $ 3.58