# Removal of *Giardia* spp. and *Cryptosporidium* spp. from Water Supply with High Turbidity: Analytical Challenges and Perspectives

Maciel, F. P. M[1] and Sabogal-Paz, L. P.[2]

[1, 2] 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.

[2] Corresponding author: 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

## ABSTRACT

*Giardia* and *Cryptosporidium* species are a serious problem if present in water supplies. The removal of these protozoans and the adaptation of existing protocols are essential for supplying drinking water to developing countries. Considering this, the aim of this study is to evaluate, on a bench level, the removal of *Giardia* spp. cysts and of *Cryptosporidium* spp. oocysts from water with high turbidity, using polyaluminium chloride as a coagulant. Filtration using mixed cellulose ester membranes, followed, or not, by purification through immunomagnetic separation was used for detecting protozoans. By evaluating the adopted protocol, without using immunomagnetic separation, retrievals of 80% of cysts and 5% of oocysts were obtained, whereas by using immunomagnetic separation, recoveries of 31.5% of cysts and 5.75% of oocysts were reached. When analyzing the coagulant performance, a dosage of 65 mg $L^{-1}$ showed contamination from protozoans in all the samples of filtered water. A dosage of 25 mg $L^{-1}$ presented protozoans in 50% of the filtered water samples. The results showed an improved performance for the 25 mg $L^{-1}$ dosage; therefore, the control of coagulation and adaptation of detection protocols must be evaluated according to the features of raw water and availability of local resources.

**Key-words:** *Cryptosporidium* spp.*; Giardia* spp.; drinking water; polyaluminium chloride.

## 1. INTRODUCTION

In spite of significant improvements in water treatment seen in recent years, there are still reports, including in developed countries, of disease transmission related to the water supply for human consumption (Braeye *et al*. 2014; Puleston *et al* 2014; Rochelle & Di Giovanni 2014; Widerström *et al*. 2014). Therefore, international agencies have turned their attention to the study and control of emerging microorganisms in light of relevant world events, mainly concerning various outbreaks of cryptosporidiosis and giardiasis (Mac Kenzie *et al*. 1994, Karanis *et al.* 2007; Baldursson & Karanis 2011).

Within this context, assessing the microbiological quality of the water supply requires removing target organisms. These organisms need to be: realistic, measurable, based on scientific data and relevant to local conditions (WHO 2011). Therefore, the *Giardia* and *Cryptosporidium* protozoans are essential for analyzing (using criteria) the drinking water supply. These protozoans appear in the environment in their form of resistance (cysts and oocysts, respectively). In order to complete their life cycle, these organisms need a host. They can cause infection in human beings, where the main symptoms are outbreaks of diarrhea. Furthermore, these organisms show high levels of persistence in aquatic environments (Olson *et al.* 1999). Usually, the presence of these protozoans in water supplies is associated to inadequate domestic sewage disposal and agricultural activities in the drainage basin.

Difficulties in removing cysts and oocysts at Water Treatment Plants (WTPs) are associated, among other factors, to physical characteristics. *Giardia* cysts have dimensions that range from 8 to 18 µm in length and from 5 to 15 µm in width, whereas the diameter of *Cryptosporidium* oocysts range between 4 and 6 µm (USEPA 2012). Given these sizes, they are potentially removable through the WTP filters, however the ability that oocysts have of compressibility (Li *et al*. 1995) can facilitate

their passage through the filtering medium, reaching, therefore, the treated water. In addition to the reduced size, the encysted forms are considerably resistant to inactivation by chlorine, a commonly used disinfectant (Korich *et al*. 1990). Thus, maximising the removal of *Giardia* and *Cryptosporidium* at WTPs is an essential issue (Brown & Emelko 2009). Studies, as the one carried out by Emelko (2003), demonstrate that coagulation failure could significantly jeopardize the removal of protozoans during the filtration process. However, there are still gaps in the scientific knowledge, such as the variables in the coagulation identified to successfully remove these parasites.

The complexity of the threat of the *Giardia* and *Cryptosporidium* species also involves difficulties in detection. Researchers worldwide have developed protocols for the evaluation of protozoa in environmental samples (Vesey *et al.* 1993; Karanis *et al.* 1998; Franco *et al.* 2001; Karanis & Kimura 2002); nevertheless, a standard procedure is required to provide credibility to results. Method 1623.1(USEPA 2012) is validated for detecting protozoans in drinking water and includes sampling, concentration, purification and detection stages using fluorescence microscopy. The possibility of modifying Method 1623.1 is already foreseen provided that the analytical quality control of the adopted protocol reaches pre-established criteria. The changes will be accepted when the performance is improved and the costs and sample processing time are reduced (USEPA 2012).

The reagents, consumption material and equipment used in Method 1623.1 represent high costs and technical analytical complexity. This fact limits the evaluation of *Giardia* and *Cryptosporidium* in drinking water supplied by Latin American water utilities, even when the law requires or enforces this monitoring. In Brazil, adaptations of Method 1623.1 are being carried out, such as the possibility of concentrating samples using mixed cellulose ester membranes as suggested by Franco *et al*. (2001). This alternative can lead to a reduction in costs, while at the same time complying with international standards. The protocol is already being used in various studies aimed at

analyzing the occurrence of *Giardia* cysts and of *Cryptosporidium* oocysts in drinking water and effluents (Franco *et al.* 2002; Neto *et al*. 2006; Nishi *et al*. 2009; Neto *et al*. 2010; Cordi *et al*. 2012). However, this protocol has not yet been tested in matrices with high turbidity.

Another possible adaptation of Method 1623.1 may be the use of methods of concentration based on flocculation procedures, according to Vesey *et al.* (1993) and Karanis & Kimura (2002). Both protocols showed high recovery for specific water samples with low number of oocysts. Nevertheless, such methods may alter the viability of cysts and oocysts with possible changes in the results of infectivity assays.

The high cost of Method 1623.1 is not restricted only to the concentration phase. Purifying the samples requires immunomagnetic separation (IMS), which can capture cysts and oocysts from a volume of a concentrated sample up to 0.5 mL. The volume purified in this procedure can be easily analyzed on microscope well slides. The possibility of eliminating the IMS, complying with international standards, could bring economic benefits to Latin American water utilities, because in Brazil the average cost of the Dynabeads® kit, used on the procedure, is US$5,000.00; one kit is sufficient for only 50 samples (quote obtained in June, 2015).

Regarding the IMS costs, sucrose-flotation methods (Karanis *et al.* 1998) can be attractive for the assess protozoa in water samples. Ramirez & Sreevatsan (2005) attained high analytical sensitivity, detecting as few as 10 oocysts in water samples. Nonetheless, Santos *et al.* (2004) obtained low recoveries in sewage samples derived from *Giardia* cyst and *Cryptosporidium* oocyst losses during the sucrose purification phase. Research on the performance of sucrose-flotation methods has been developed in Latin America (Quintero-Betancourt & Ledesma, 2000; Bonatti *et al.* 2007), however, more studies that include water matrix characteristics and availability of local resources must be performed.

The application of a simplified protocol, which complies with the restrictions of Method 1623.1 in Latin America, will make it possible to estimate the dynamics of these parasites in water, and therefore, measures can be taken to improve the efficiency of the treatment phases aiming to provide good quality water to the population.

Along these lines, this study assessed the performance of the method by Franco *et al*. (2001), followed, or not, by IMS, aiming to analyze the possibility of reducing the complexity and costs involved in the protocol for detecting *Giardia* spp. cysts and *Cryptosporidium parvum* oocysts. The study also considered the removal of these parasites in Jar-Test assays using polyaluminium chloride for treating water with high turbidity and the presence of protozoans.

## 2. MATERIALS AND METHODS

The study was carried out in three steps. In Step 1, Jar-test assays were carried out to make coagulation diagrams in order to select the optimal points (coagulant dosage vs. pH coagulation, with and without pH correction). Then, the parameters concerning the treatment (rapid-mix, slow-mix, and sedimentation velocity) were optimized. The polyaluminium chloride (PACl) had an alkalinity of 38.7, $Al_2O_3$ content of 17.74% and a pH value of around 3. For the filtration, laboratory filters (LF) were used, filled with sand grains (size range from 0.30 to 0.59 mm), which were operated at the filtration rate of 100 $m^3\ m^2\ d^{-1}$. The efficiency of the treatment was evaluated by the filtered water's turbidity. The water from the study, without protozoans, was prepared by adding kaolinite to a water well, at a proportion of 0.1 $g\ L^{-1}$, necessary for obtaining turbidity close to 125 NTU. This study aimed at assessing the performance of the protocol adopted for *Giardia* cysts and *Cryptosporidium* oocysts; therefore, a synthetic water sample was prepared for the elimination of possible interferences in real water samples. Obviously, each laboratory protocol must be adapted to

the characteristics of the matrix under study. The physical and chemical analyses carried out followed the procedures described in APHA *et al.* (2012).

In Step 2, the analytical quality control method by Franco *et al.* (2001) was performed with adaptations associated to the volume of the sample being processed. Therefore, a known number of cysts and oocysts were inoculated in 1 L of the water from the study. *EasySeed*® and *ColorSeed*® were used with the same aim because the water from the study lacked protozoans. The inoculation was carried out according to the manufacturer´s instructions. The water from the study with protozoans was processed according to the method by Franco *et al.* (2001), with and without IMS.

Although methods of concentration, such as calcium carbonate flocculation (Vesey *et al.* 1993) and ferric sulfate flocculation (Karanis & Kimura, 2002), could be tested in this study, the protocol of Franco *et al.* (2001) was assessed because it is easier and faster in comparison with flocculation procedures and the viability of cysts and oocysts can be preserved for future infectivity assays.

The well slide was prepared using the kit from Merifluor® and then scanned using fluorescence microscopy. The number of cysts and oocysts found were compared to the number that was inoculated. Therefore, from the data obtained, the mean recovery percentage and the variation coefficient were estimated and compared to the standard of Method 1623.1.

In Step 3, the performance of the treatment was evaluated (coagulation, flocculation, sedimentation and filtration), simulated in the Jar-test to remove cysts and oocysts, using the results obtained during Step 1 and Step 2. During this Step, the water from the study was inoculated with approximately 500 cysts $L^{-1}$ of *Giardia* spp. and 500 oocysts $L^{-1}$ of *Cryptosporidium parvum* in each Jar-test jar; each jar was previously rinsed using *Tween* 80 (0.1%). The cysts were purified at the Protozoology Laboratory at the State University of Campinas (Campinas/SP, Brazil) and the

oocysts were acquired from Waterborne (USA). The estimate of the number of cysts and oocysts was carried out by evaluating 5 µL of the homogenized concentrated solutions, in triplicate, using the *Merifluor*® kit. The mean value of the readings enabled the quantification of the target organisms present in a determined volume of strain to be inoculated. The aggregation of oocysts, which made it difficult to count, was eliminated by diluting the strain in *Tween* 20 (0.01%) with subsequent homogenization using a test tube (20 times). Afterwards, the mixture was centrifuged at 1,500 x *g* for 10 min disposing the supernatant with a subsequent introduction of PBS until the level of 1 mL.

When applying the method by Franco *et al.* (2001), a vacuum pump was used to filter the samples in mixed cellulose ester membranes (diameter of 47 mm and nominal porosity of 3 µm). Filtration was maintained with a leakage ranging from 1 to 4 L $min^{-1}$ and a pressure of 525 mm Hg. Before being in contact with protozoans, all equipment and glassware were rinsed using *Tween*® 80 (0.1%). Filtration was carried out until dripping started, always aiming to avoid exceeding the pressure of 600 mm Hg and, at that point in time, the membrane was changed if necessary. After that, the membranes were carefully removed using metal tweezers and then transferred to a tilted *Petri* dish, from where the retained material was scraped and washed. Scraping was done using a soft plastic strap in parallel movements followed by washing it using 1.0 mL of *Tween* 80 (0.01%). The yielding liquid was homogenized using a 3.0 mL *Pasteur* test tube and then transferred to a centrifuge tube. The procedure was repeated twice, keeping the membrane turned at 90º from the previous scrapings. The centrifuge tube was filled with *Tween* 80 up to 50 mL and then centrifuged at 1,500 x *g* for 15 min. After the first centrifugation, the pellet was formed, and then, the supernatant was vacuumed leaving 10 mL of sediment in the tube. After this, the liquid was homogenized in vortex and filled up with ultrapure water to submit it to another centrifugation for the same time and at the same rotation rate.

The sample assessed (without IMS) considered disposing of the supernatant after the second centrifugation, leaving a final sediment of 1,000 µL (1.0 mL). The sediment was carefully homogenized before removing the aliquots for disposal on glass well slides. During the procedure, 50 µL of the sediment was divided into 5 glass wells to analyze the sediment residue and the water from the study and the sediment water. Regarding the filtered water, 75 µL was placed in a single glass well.

The analyzed sample (with IMS) included disposing of the supernatant after the second centrifugation, leaving a sediment of at least 5,000 µL (5.0 mL), depending on the estimated volume of the pellet. The guidelines established in Method 1623.1 were followed when the volume of the pellet was higher than 500 µL. The IMS was processed to capture cysts and oocysts according to the *Kit Dynabeads*® manufacturer´s instructions by two acid dissociations, aiming to separate the target microorganisms from the magnetic microspheres and then, the slide was prepared using the *Merifluor*® kit. During the procedure, regardless of the sample, the volume transferred to the slide was 100 µL (50 µL at each well considering the two acid dissociations).

The slide was read at a magnification of x 200 for identification, and was confirmed at a magnification of up to x 800. Morphological confirmatory aspects were observed by DAPI staining and DIC microscopy, at a magnification of x 400. The criteria of Method 1623.1 were followed for characterization of the target organisms. After identification and enumeration, Equation 1 was applied.

$$E = \frac{NxS}{VxA} \times FC \qquad (1)$$

Where, E: estimate of number of protozoans (cysts $L^{-1}$ or oocysts $L^{-1}$); N: number of cysts or oocysts observed in the well(s) of the immunofluorescence slide; S: volume of final sediment (µL); V: total volume of the analyzed sample (L); A: volume of the aliquot transferred to the well of the slide (µL); FC: correction factor only for samples of sediment residue (total volume / concentrated volume).

The performances of the protocol by Franco *et al.* (2001) and of the simulation treatment in the Jar-test (with and without IMS) were weighed according to analysis of variance (ANOVA test).

## 3. RESULTS AND DISCUSSION

Table 1 shows the optimal conditions of the studied water treatment, which was able to generate filtered water with turbidity below 0.5 UNT.

Table 1. Parameters Obtained from the Jar-test Assays

| Parameter | Without pH adjustment | With pH adjustment |
|---|---|---|
| Dosage of PACl (mg $L^{-1}$) | 25 | 65 |
| pH coagulation | 6.8 | 7.2 |
| Mean velocity gradient for rapid mixing ($s^{-1}$) | 900 (400 rpm) | 1000 (450 rpm) |
| Rapid mixing time (s) | 15 | 15 |
| Mean velocity gradient for flocculation ($s^{-1}$) | 30 (40 rpm) | 20(30 rpm) |
| Flocculation time (min) | 20 | 20 |
| Sedimentation velocity (cm $min^{-1}$) | 1.5 | 1.5 |
| Filtration rate ($m^3$ $m^{-2}$ $d^{-1}$) | 100 | 100 |

Characteristics of the water from the study: total alkalinity = 36 mg $CaCO_3$ $L^{-1}$; total aluminium = 0.91 mg $L^{-1}$; total coliforms = 6 NMP 100 $mL^{-1}$; *Escherichia coli* = absent; electrical conductivity = 53.7 µS $cm^{-1}$; hardness = 14 mg $CaCO_3$ $L^{-1}$; total iron = 0.037 mg $L^{-1}$; total manganese = 0.008 mg $L^{-1}$; nitrate = 0.05 mg $L^{-1}$; nitrite = 0.005 mg $L^{-1}$; and turbidity = 125 UNT.

Characteristics of the filtered water: total alkalinity = 20 mg $CaCO_3$ $L^{-1}$; total aluminium < 0.001 mg $L^{-1}$; total coliforms = absent; *Escherichia coli* = absent; electrical conductivity = 70.8 µS $cm^{-1}$; hardness = 14 mg $CaCO_3$ $L^{-1}$; total iron < 0.005 mg $L^{-1}$; total manganese < 0.003 mg $L^{-1}$; nitrate < 0.01 mg $L^{-1}$; nitrite < 0.001 mg $L^{-1}$; and turbidity < 0.5 UNT.

The results of the assays for analytical quality control of the method by Franco *et al*. (2001) are shown in Table 2. The recovery (with and without IMS) did not comply with the requirements of Method 1623.1 for *Cryptosporidium parvum* oocysts.

Table 2. Results of the Analytical Quality Assays of the Method by Franco *et al*. (2001) (with and without IMS) for Water from the Study

| Method | Recovery mean percentage (number of assays = 4) | | Variation Coefficient (number of assays = 4) | |
|---|---|---|---|---|
| | *Giardia* spp. | *Cryptosporidium parvum* | *Giardia* spp. | *Cryptosporidium parvum* |
| Without IMS | 80.0% | 5.0% | 20.4% | 200.0% |
| With IMS | 31.5% | 5.7% | 24.0% | 55.7% |

The loss of cysts and oocysts could be related to the scraping and centrifugation phases. The method tested considered centrifugation at 1,500 x *g* for 15 min; however, Clancy *et al*. (2000) pointed out that values of 2,170 x g for 10 min could improve the recovery. Nevertheless, Method 1623.1 recommends values in the range from 1,500 x *g* to 2,000 x *g*, with reservations to the highest rotations in samples with sediments, as there is the possibility of damaging the target organisms. Another possibility is the difficulty to view oocysts through the microscope, since the fluorescent dye can fade when using the Merifluor® kit. The same problem was reported by Clancy *et al*. (2000) when assessing seven commercial brands. Intermediate assays for quality control of the method were carried out following guidelines by USEPA (2012) and the results obtained were similar to those shown in Table 2.

The assays (without IMS) resulted in high recovery efficiencies of *Giardia* spp. cysts (Table 2). The procedure considered the reading of 50 µL of the sample, divided into 5 wells, from 1,000 µL of sediment. Thus, the organism observed corresponded to 20 organisms of the concentrated sample. This high multiplication factor should be evaluated carefully based on a possible mistake of the estimate. Another aspect that should be considered is the quality of the image viewed through the

microscope. The sample presented dense sediment for the 10 µL taken to the well of the slide. This fact created a barrier that made it difficult to view cysts and oocysts and also made the analysis impossible by DIC microscopy and DAPI fluorescence. Based on this, the analysis of the samples that were not submitted to IMS remained restricted to the FITC microscopy. This factor represents a limitation of the method without IMS, since algae or other microorganisms could be counted as protozoans if presenting fluorescence, size and pattern of compatible membranes.

The assays (with IMS) showed a better view of the protozoans through the microscope. Considering this, morphological aspects could be confirmed by eliminating the sample impurities (color by DAPI and visualization by DIC). IMS is advantageous because the sample can be thoroughly analyzed, and therefore the multiplication factor is eliminated. Nevertheless, the procedure entails introducing various steps for the detection protocol, and therefore, when choosing it, the characteristics of the sample, the time of analysis and its economic viability should be considered. Obviously, the knowledge on the matrix characteristics is important for the evaluation of the best protocols to be selected. In complex matrices, sucrose-flotation methods may be an option for the purification of the samples, however, such an assumption must be tested.

The removal of protozoans, by Jar-test assays, remained in the range of 2 $Log_{10}$, according to Table 3, which is compatible with Haas *et al*. (2001). Assavasilavasukul *et al*. (2008) reported removals ranging from 0.73 to 5.2 $Log_{10}$ for *Cryptosporidium* spp. and from 1.0 to 5.4 $Log_{10}$ for *Giardia* spp.. These results were obtained by treating water through coagulation, flocculation, sedimentation and filtration. Changes in the concentration of protozoans of the effluent, in the turbidity of the raw water and in the sampling methods contributed to the differences in the observed removals. By analyzing the data obtained, which is presented in Table 3, no statistical difference was observed between the two conditions of coagulation studied by the ANOVA test, based on a reliability level of 95%.

Table 3. Removal of *Giardia* spp. cysts and *C.parvum* oocysts using Jar-Test assays

| Method | Protozoan | Removal in $Log_{10}$ | |
|---|---|---|---|
| | | 25 mg $L^{-1}$ of PACl (number of assays = 4) | 65 mg $L^{-1}$ of PACl (number of assays = 3) |
| Without IMS | *Giardia* spp. | 2.26 ± 0.14 | 2.31 ± 0.26 |
| With IMS | *Giardia* spp. | 3.10 ± 0.41 | 2.93 ± 0.06 |
| Without IMS | *C. parvum* | 1.31 ± 0.35 | 1.71 ± 0.38 |
| With IMS | *C. parvum* | 2.13 ± 0.44 | 2.19 ± 0.10 |

Table 4 shows some variables involved in the coagulation and characteristics, concerning protozoans, for the water from the study and for the filtered water. The results indicated that the method (without IMS) had a low sensitivity to detect parasites in the filtered water. However, the method (with IMS) for the filtered water, resulting in the dosage of 25 mg $L^{-1}$ of PACl, showed 50% of the samples as positive for cysts and oocysts, whereas for the 65 mg $L^{-1}$ dosage, 100% of the samples presented protozoans.

Table 4. Some coagulation variables and characteristics of water from the study and filtered water concerning protozoans

| Dosage | Coagulation variables | | Turbidity (UNT) | | *Giardia* spp. | | | | *Cryptosporidium parvum* | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | (cysts.$L^{-1}$) | | | | (oocysts.$L^{-1}$) | | | |
| | pH | Zeta potential (mV)[1] | WS | FW | With IMS | | Without IMS | | With IMS | | Without IMS | |
| | | | | | WS | FW | WS | FW | WS | FW | WS | FW |
| 25 mg $L^{-1}$ of PACl (number of assays = 4) | 6.70 | 8.68 | 130 | 0.19 | 576 | <1 | 695 | ND | 12 | ND | 17 | ND |
| | 6.88 | 4.74 | 125 | 0.25 | 512 | <1 | 532 | ND | 14 | ND | 81 | ND |
| | 6.77 | 8.29 | 125 | 0.30 | 180 | ND | 517 | ND | 111 | ND | 64 | ND |
| | 6.70 | 6.87 | 132 | 0.25 | 79 | ND | 317 | ND | 62 | <1 | 100 | ND |
| Mean | 6.76 | 7.15 | 128 | 0.25 | 337 | | 515 | | 50 | | 65 | |
| Standard deviation | 0.08 | 1.78 | 3.5 | 0.05 | 244 | | 155 | | 47 | | 35 | |
| 65 mg $L^{-1}$ de PACl (number of assays = 3) | 7.07 | -13.60 | 125 | 0.32 | 164 | <1 | 333 | ND | 32 | <1 | 50 | ND |
| | 7.40 | -16.60 | 126 | 0.25 | 416 | <1 | 450 | <2 | 37 | <1 | 200 | <2 |
| | 7.40 | -14.10 | 134 | 1.0 | 272 | <1 | 1067 | ND | 252 | <1 | 250 | ND |
| Average | 7.29 | -14.77 | 128 | 0.52 | 284 | | 617 | | 107 | | 167 | |
| Standard deviation | 0.19 | 1.61 | 4.9 | 0.41 | 126 | | 394 | | 126 | | 104 | |

[1] Zeta potential of water from the study = -21.8 mV; ND = non-detected; WS: water from the study; FW: filtered water.

By assessing Table 4, it can be observed that the pH coagulation is lower for the dosage of 25 mg $L^{-1}$ of PACl. According to Hsu *et al*. (2001), Hsu & Huang (2002) and Tufenkji *et al*. (2006), an increase is expected in the removal of *Cryptosporidium* oocysts by filtering water at a low pH level, since in this condition, it is believed that the zeta potential of oocysts will be less electronegative, therefore favouring its retention. Following this assumption, it is thought that the addition of alkalizing, necessary for the 65 mg $L^{-1}$ of PACl dosage, could have been harmful to the removal of protozoans in the filtering medium. Fernandes *et al*. (2010), assessed the direct filtration technology on a pilot scale, and also observed an increase in the removal of *Cryptosporidium* oocysts in a pH value around 5. Bustamante *et al.* (2001) measured the zeta potential of flakes, oocysts and the oocysts/flakes conjugate with different pH values, when aluminium sulphate was applied (6.8 mg Al $L^{-1}$). They observed that the zeta potential was positive for the pH, in the range between 4 and 6.8 with a severe decline in the value as the pH was increased (Figure 1).

According to Table 4, dosages of 25 mg $L^{-1}$ and 65 mg $L^{-1}$ of PACl presented a zeta potential of 7.15 ± 1.78 mV in pH values of 6.76 ± 0.08 and of -14.77 ± 1.61 in pH of 7.29 ± 0.19, respectively. The results warn us that, in conditions close to neutral pH, the coagulation zeta potential is similar to that of the cysts/oocysts/flake conjugate found by Bustamante *et al.* (2001), as depicted in Figure 1. Apparently, the reversal of loads, at the point of coagulation, could be the answer to the best performance shown by the 25 mg $L^{-1}$ of PACl dosage, since this could have favoured the cysts/oocysts/flake conjugate's structure and its retention in the filtrating medium. It is highlighted that the measurement of the zeta potential cysts/oocysts' surface is not practical, in contrast to the solution's zeta potential measurement, as was carried out in this study.

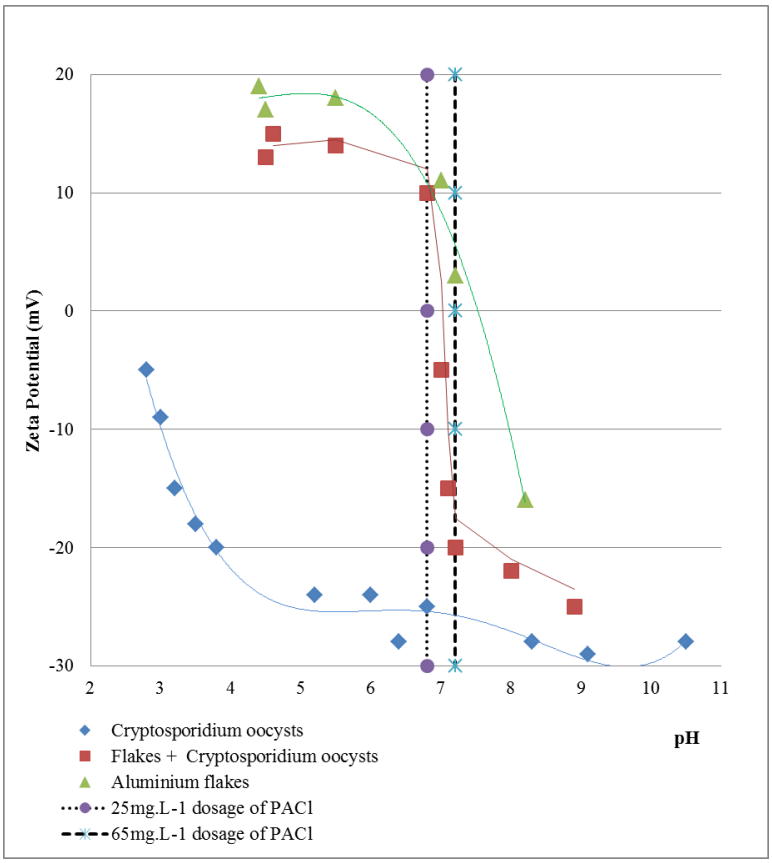


Figure 1. Effect of pH on the zeta potential of the flakes, oocysts and oocysts/flakes conjugate (Bustamante *et al.* 2001). The vertical lines represent the pH coagulation for the 25 mg $L^{-1}$ and 65 mg $L^{-1}$ dosages analyzed in this study.

In the study, the relevance of the two consecutive acid dissociations, for the method using IMS, was clear (Table 5). The results showed that the second dissociation, when compared to the first one, presented a higher recovery of *Giardia* spp. cysts according to ANOVA test, at a significance level of 95%. The same behaviour was not observed for *Cryptosporidium parvum* oocysts. In spite of this, and regardless of the prevalence of the first or second dissociation, it is essential that this procedure be carried out in two steps.

Table 5. Percentage distribution of the recovery of cysts and oocysts between the two acid dissociations of IMS

| Dosage | Samples | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Raw water | | Settled water | | Filtered water | | Sedimented residue | |
| | Dissociations (results for *Giardia spp.* cysts) | | | | | | | |
| | **1st** | **2nd** | **1st** | **2nd** | **1st** | **2nd** | **1st** | **2nd** |
| 25 mg $L^{-1}$ of PACl (number of assays = 4) | 36% | 64% | 0% | 100% | 0% | 100% | 24% | 76% |
| | 19% | 81% | 89% | 11% | 100% | 0% | 90% | 10% |
| | 45% | 55% | 0% | 0% | 0% | 0% | 56% | 44% |
| | 43% | 57% | 0% | 0% | 0% | 0% | 6% | 94% |
| 65 mg $L^{-1}$ of PACl (number of assays = 3) | 12% | 88% | 62% | 38% | 0% | 100% | 29% | 71% |
| | 58% | 42% | 50% | 50% | 100% | 0% | 69% | 31% |
| | 15% | 85% | 0% | 100% | 0% | 100% | 53% | 47% |
| Mean | 32% | 68% | 40% | 60% | 40% | 60% | 47% | 53% |
| Standard deviation | 17% | 17% | 39% | 39% | 55% | 55% | 29% | 29% |
| Dosage | Dissociations (results for oocysts of *Cryptosporidium parvum*) | | | | | | | |
| | **1st** | **2nd** | **1st** | **2nd** | **1st** | **2nd** | **1st** | **2nd** |
| 25 mg $L^{-1}$ of PACl (number of assays = 4) | 62% | 38% | 0% | 0% | 0% | 0% | 0% | 100% |
| | 53% | 47% | 0% | 100% | 0% | 0% | 100% | 0% |
| | 86% | 14% | 0% | 0% | 0% | 0% | 0% | 0% |
| | 67% | 33% | 0% | 0% | 100% | 0% | 17% | 83% |
| 65 mg $L^{-1}$ of PACl (number of assays = 3) | 59% | 41% | 100% | 0% | 100% | 0% | 0% | 100% |
| | 43% | 57% | 0% | 0% | 0% | 100% | 0% | 0% |
| | 46% | 54% | 0% | 0% | 100% | 0% | 0% | 100% |
| Mean | 59% | 41% | 50% | 50% | 75% | 25% | 23% | 77% |
| Standard deviation | 14% | 14% | 71% | 71% | 50% | 50% | 43% | 43% |

$1^{st}$ = first acid dissociation; and $2^{nd}$ = second acid dissociation.

The data in Table 5 suggests a strong interaction between the cysts and the Dynabeads® Anti-Giardia microspheres, a phenomenon that can be viewed through the microscope by observing cysts still adhered to microspheres after the acid dissociation (Figure 2). This event was not detected for *Cryptosporidium parvum* oocysts.

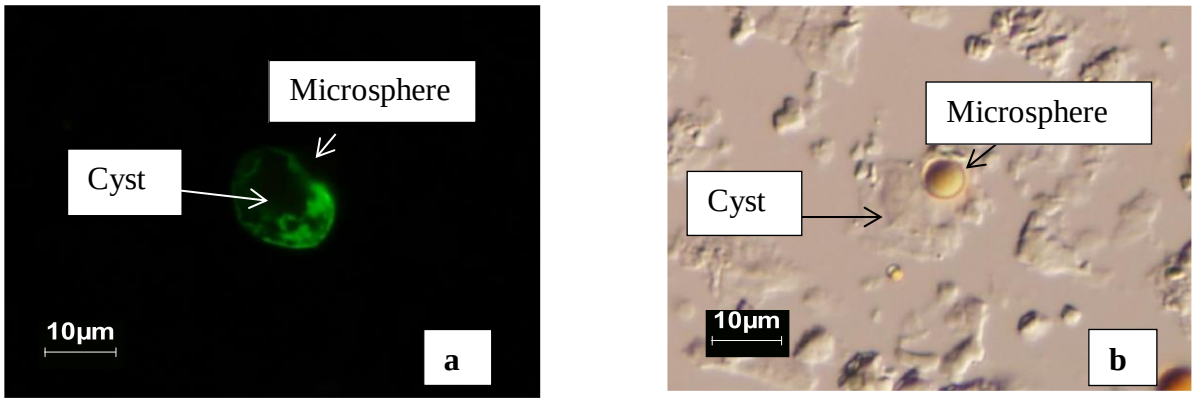


Figure 2. *Giardia* spp. cyst adhered to the microsphere, at an increase of 400X, viewed in FITC (a) and DIC (b)

The cost of reagents per sample, considering only the Merifluor® and Dynabeads® kits, was estimated at US$ 198 and US$ 190 for assays with and without IMS, respectively (quote in June, 2015). Therefore, the use of IMS increased the costs by approximately 4%.

To sum up, the decision regarding using the method by Franco *et al*. (2001) (with and without IMS), should be based on: the presence of *Cryptosporidium parvum* oocysts in the raw water; the quality of the water sample; the volume of the sample being processed; the time needed for laboratory analysis and the costs of the reagents, equipment and qualified workmanship.

## 5. CONCLUSIONS

Polyaluminium chloride, when used to treat water with high turbidity, enabled us to generate filtered water with turbidity lower than 0.5 UNT. However, *Giardia* spp. cysts and *Cryptosporidium parvum* oocysts were detected in the filtered water. Thus, turbidity is not a variable recommended to control protozoans in drinking water.

The protocol by Franco *et al*. (2001) (with and without IMS), met the standard of Method 1623.1 only for *Giardia* spp. cysts and the results showed the importance of the second acid dissociation to assess this parasite. Obviously, other simplified methodologies need to be tested aiming to detect *Cryptosporidium parvum* oocysts in environmental samples in Latin America.

The removal of protozoans, tested by the Jar-test, remained around 2 $Log_{10}$, and, apparently there is a better performance for the dosage of 25 mg $L^{-1}$ of PACl, with positive zeta potential, when treating water that has high turbidity and a presence of protozoans.

## 6. ACKNOWLEDGEMENTS

The authors are grateful to National Council for Scientific and Technological Development (CNPq) for the masters scholarship awarded to Paulo Marcos Faria Maciel and São Paulo Research Foundation (FAPESP) for the research support (Process 2012/50522-0).

## 6. BIBLIOGRAPHY

American Public Health Association – APHA, American Water Works Association – AWWA, Water Environment Federation – WEF (2012). Standard methods for the examination of water and wastewater. Washington, DC: American Public Health Association.

Assavasilavasukul, P., Lau, B. L., Harrington, G. W., Hoffman, R. M., & Borchardt, M. A. (2008). Effect of pathogen concentrations on removal of Cryptosporidium and Giardia by conventional drinking water treatment. *Water Res*. **42** (10), 2678-2690.

Baldursson, S., & Karanis, P. (2011). Waterborne transmission of protozoan parasites: review of worldwide outbreaks–an update 2004–2010. *Water Res.* **45** (20), 6603-6614.

Bonatti, T. R., Franco, R. M. B, Cantusio Neto, R. (2007) Comparison of two methodologies for detection of *Giardia* spp. cysts and *Cryptosporidium* spp. oocysts in activated sludge samples from a sewage treatment plant in the city of Campinas, São Paulo State Brazil. *J Water Health.* **5** (4), 609-614.

Braeye, T., De Schrijver, K., Wollants, E., Van Ranst, M., & Verhaegen, J. (2014). A large community outbreak of gastroenteritis associated with consumption of drinking water contaminated by river water, Belgium, 2010. *Epidemiol Infect*. **143** (04), 711-719.

Brown, T. J., & Emelko, M. B. (2009). Chitosan and metal salt coagulant impacts on *Cryptosporidium* and microsphere removal by filtration. *Water Res*. **43** (2), 331-338.

Bustamante, H. A., Shanker, S. R., Pashley, R. M., & Karaman, M. E. (2001). Interaction between *Cryptosporidium* oocysts and water treatment coagulants. *Water Res*. **35** (13), 3179-3189.

Clancy, J. L., Bukhari, Z., McCuin, R. M., Hargy, T. M., Fricker, C. R., Matheson, Z., & Sykes, N. (2000). *New approaches for isolation of Cryptosporidium and Giardia*. In New approaches for isolation of *Cryptosporidium* and *Giardia*. American Water Works Association.

Cordi, L., Lima, P., França, R. B., Franco, R. M. B., & Durán, N. (2012). Activated Sludge and Ozonation Combined System of Sanitary Effluent Treatment to Bacterial and Protozoa Removal-A Case Study. *Energy and Environment Res*. **2** (2), p149.

Emelko, M. B. (2003). Removal of viable and inactivated *Cryptosporidium* by dual-and tri-media filtration. *Water Res*. **37** (12), 2998-3008.

Fernandes, N. M. G., Ginoris, Y. P., Rios, R. H. T., & Brandão, C. C. S. (2010). The influence of coagulation pH and aluminum sulphate dose in removal of *Cryptosporidium* oocysts by down flow direct filtration. *Eng Sanit Ambient*. **15** (4), 375-384.

Franco, R. M. B., Rocha-Eberhardt, R., & Cantusio Neto, R. (2001). Occurrence of *Cryptosporidium* oocysts and *Giardia* cysts in raw water from the Atibaia river, Campinas, Brazil. *Rev Inst Med Trop São Paulo*. **43** (2), 109-111.

Franco, R. M. B., & Cantusio Neto, R. (2002). Occurrence of *Cryptosporidium* oocysts and *Giardia* cysts in bottled mineral water commercialized in the City of Campinas, State of Sao Paulo, Brazil. *Mem Inst Oswaldo Cruz*. **97** (2), 205-207.

Hass, C., French, K., Finch, G.R., Guest, R.K., 2001. Data review on the physical/chemical removal of Cryptosporidium. AWWA Research Foundation, Denver, Co. http://www.waterrf.org/PublicReportLibrary/90834.pdf. (accessed June 25, 2015).

Hsu, B. M., & Huang, C. (2002). Influence of ionic strength and pH on hydrophobicity and zeta potential of *Giardia* and *Cryptosporidium*. *Colloid Surface A*. **201** (1**)**. 201-206.

Hsu, B. M., Huang, C., & Pan, J. R. (2001). Filtration behaviors of *Giardia* and *Cryptosporidium* ionic strength and pH effects. *Water Res*. **35** (16), 3777-3782.

Karanis, P., Schoenen, D., & Seitz, H. M. (1998). Distribution and removal of *Giardia* and *Cryptosporidium* in water supplies in Germany. *Water Sci Technol*. **37** (2), 9-18.

Karanis, P., & Kimura, A. (2002). Evaluation of three flocculation methods for the purification of *Cryptosporidium parvum* oocysts from water samples. *Lett Appl Microbiol*. **34** (6), 444-449.

Karanis, P., Kourenti, C., & Smith, H. (2007). Waterborne transmission of protozoan parasites: a worldwide review of outbreaks and lessons learnt. *J Water Health*. **5** (1), 1-38.

Korich, D. G., Mead, J. R., Madore, M. S., Sinclair, N. A., & Sterling, C. R. (1990). Effects of ozone, chlorine dioxide, chlorine, and monochloramine on *Cryptosporidium parvum* oocyst viability. *Appl Environ Microb*. **56** (5), 1423-1428.

Li, S. Y., Goodrich, J. A., & Owens, J. H. (1995). Potential *Cryptosporidium* surrogates and evaluation of compressible oocysts (No. CONF-9504110--). Environmental Protection Agency, Cincinnati, OH (United States). http://infohouse.p2ric.org/ref/37/36916.pdf (accessed April 22, 2015).

Mac Kenzie, W. R., Hoxie, N. J., Proctor, M. E., Gradus, M. S., Blair, K. A., Peterson, D. E., ... & Davis, J. P. (1994). A massive outbreak in Milwaukee of *Cryptosporidium* infection transmitted through the public water supply. *New Engl J Med*. **331** (3), 161-167.

Neto, R. C., Santos, J. U., & Franco, R. M. B. (2006). Evaluation of activated sludge treatment and the efficiency of the disinfection of *Giardia* species cysts and *Cryptosporidium* oocysts by UV at a sludge treatment plant in Campinas, south-east Brazil. *Water Sci Technol*. **54** (3), 89-94.

Neto, R. C., dos Santos, L. U., Sato, M. I., & Franco, R. M. (2010). *Cryptosporidium* spp. and *Giardia* spp. in surface water supply of Campinas, southeast Brazil. *Water Sci Technol* . **62** (1),217–222. IWA Publishing 2010

Nishi, L., Bergamasco, R., Toledo, M. J. D. O., Falavigna, D. L. M., Gomes, M. L., Mota, L. T., & Falavigna-Guilherme, A. L. (2009). *Giardia* spp. and *Cryptosporidium* spp. in the Ivaí indigenous land, Brazil. *Vector Borne Zoonotic Dis*. **9** (5), 543-547.

Olson, M. E., Goh, J., Phillips, M., Guselle, N., & McAllister, T. A. (1999). *Giardia* cyst and *Cryptosporidium* oocyst survival in water, soil, and cattle feces. *J Environ Qual*. **28** (6). 1991-1996.

Puleston, R. L., Mallaghan, C. M., Modha, D. E., Hunter, P. R., Nguyen-Van-Tam, J. S., Regan, C. M., ... & Chalmers, R. M. (2014). The first recorded outbreak of cryptosporidiosis due to *Cryptosporidium cuniculus* (formerly rabbit genotype), following a water quality incident. *J Water Health*. **12** (1). 41-50.

Quintero-Betancourt, W. & Ledesma, L. B. (2000). Descriptive study on the presence of protozoan cysts and bacterial indicators in a drinking water treatment plant in Maracaibo, Venezuela**.** *Int J Environ Heal R.* **10**, 51–61.

Ramirez, N. E., & Sreevatsan, S. (2006). Development of a sensitive detection system for *Cryptosporidium* in environmental samples. *Vet Parasitol*. ***136*** (3)**,** 201-213.

Rochelle, P. A., & Di Giovanni, G. D. (2014). *Cryptosporidium* oocysts in drinking water and recreational water. In *Cryptosporidium: parasite and disease* (pp. 489-513). Springer Vienna.

Santos, L.U., Bonatti T.R., Cantusio Neto, R., Franco R. M. B (2004). Occurrence of *Giardia* cysts and *Cryptosporidium* oocysts in activated sludge samples in Campinas, SP, Brazil. *Rev Inst Med Trop São Paulo*. **46**, 309–313

Tufenkji, N., Dixon, D. R., Considine, R., & Drummond, C. J. (2006). Multi-scale *Cryptosporidium*/sand interactions in water treatment. *Water Res.* **40** (18), 3315-3331.

United States Environmental Protection Agency – USEPA (2012). Method 1623.1 *Cryptosporidium* and *Giardia* in Water by Filtration/IMS/FA. Office of Water (MS-140) EPA 816-R-12-001. http://water.epa.gov/scitech/drinkingwater/labcert/upload/epa816r12001.pdf (accessed January 4, 2015).

Vesey, G., Slade, J. S., Byrne, M., Shepherd, K., & Fricker, C. R. (1993). A new method for the concentration of Cryptosporidium oocysts from water. *J Appl Bacteriol*. **75** (1), 82-86.

Widerström, M., Schönning, C., Lilja, M., Lebbad, M., Ljung, T., Allestam, G., ... & Lindh, J. (2014). Large outbreak of *Cryptosporidium hominis* infection transmitted through the public water supply, Sweden. *Emerg Infect Dis*. **20** (4**)**. 581.

World Health Organization – WHO (2011). Guidelines for drinking-water quality. 4th ed. http://whqlibdoc.who.int/publications/2011/9789241548151_eng.pdf (accessed June 25, 2015).