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Use of an acrylamide-based anionic polymer for the concentration and removal of *Giardia duodenalis* cysts from high-turbidity water samples

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ABSTRACT

The increasing investigation into environmental contamination by *Giardia* spp. cysts and its impact on public health has spurred interest in developing more sensitive methods for detecting these protozoa, particularly in water. However, these methods remain complex and costly. This study evaluated the concentration and removal efficiency of *Giardia duodenalis* cysts using an acrylamide-based anionic flocculant polymer (AFP) in filter backwash water samples from a water treatment plant (WTP) located in Blumenau City, southern Brazil, and compared them with the calcium carbonate flocculation technique (CCF), which is routinely used for water matrices with high turbidity. The average recovery rates of *G. duodenalis* cysts using CCF and AFP were 33.33% and 43.33%, respectively, with an average turbidity reduction of 98.39% and 98.78%. The use of an anionic flocculant polymer proved to be an efficient alternative for the concentration and removal of protozoan cysts in water samples with high turbidity. It is important to highlight that the development and application of new studies and strategies aimed at increasing the efficiency of the removal of these organisms from complex environmental matrices would bring benefits to public health and promote a One Health perspective.

Keywords: cyst recovery, high turbidity, water.

Utilização de um polímero aniônico à base de acrilamida como uma estratégia útil para concentração e remoção de cistos de *Giardia duodenalis* em amostras de água com alta turbidez

RESUMO

A crescente investigação da contaminação ambiental por cistos de *Giardia* spp. e seu impacto na saúde pública despertou o interesse em desenvolver métodos mais sensíveis para detectar esses protozoários, especialmente na água. No entanto, esses métodos ainda são complexos e dispendiosos. Este estudo teve como objetivo avaliar a eficiência de concentração e remoção de cistos de *Giardia duodenalis* usando um polímero floculante aniônico à base de acrilamida (AFP) em amostras de água de retrolavagem de filtro de uma estação de tratamento de água (ETA) localizada na cidade de Blumenau, sul do Brasil, e compará-lo com a técnica de floculação de carbonato de cálcio (CCF), rotineiramente usada em matrizes de água com alta turbidez. A taxa de recuperação média de cistos de *G. duodenalis* usando CCF e AFP foi de 33,33% e 43,33%, com uma redução média de turbidez de 98,39% e 98,78%, respectivamente. O uso de um polímero floculante aniônico provou ser uma alternativa eficiente para a concentração e remoção de cistos de protozoários em amostras de água com alta turbidez. É importante destacar que o desenvolvimento e a aplicação de novos estudos e estratégias que aumentem a eficiência da remoção desses organismos de matrizes ambientais complexas trariam benefícios tanto do ponto de vista da saúde pública quanto da perspectiva da saúde única.

Palavras-chave: água, alta turbidez, recuperação de cistos.

1. INTRODUCTION

Water is a crucial resource for life, playing a significant role in a variety of biological processes. Anthropogenic impacts, together with new emerging pollutants, are a growing worldwide concern (Basheer, 2018).

Moreover, lack of access to basic sanitation exacerbates this situation, increasing the contamination of multiple water environments and maximizing the risk of dissemination of waterborne diseases, such as those caused by protozoa (Fradette *et al.*, 2022; Leal *et al.*, 2024).

Giardia duodenalis infections are more frequently detected in regions with poor sanitation, although waterborne outbreaks are more frequently reported in developed countries. Giardiasis may be acquired by different transmission routes; however, ingestion of contaminated water is considered the most relevant path of infection, especially for drinking water or during recreational water activities (Ongerth *et al.*, 2018; Barragán *et al.*, 2022; Zhang *et al.*, 2022).

Due to the increasing incidence of waterborne outbreaks around the world, the investigation of environmental contamination by *G. duodenalis* cysts and the impact on public health has led to the development of more sensitive detection methods, however, they are still complex and costly (Baldurson and Karanis, 2011; Efstratiou *et al.*, 2017; Ma *et al.*, 2022; Fradette *et al.*, 2022).

Method 1623.1, established by the United States Environmental Protection Agency (USEPA), is the reference method for detecting *Giardia* spp. in water, although it may present limited effectiveness in high-turbidity water samples. Other factors, such as greater laboratory complexity, execution time, and specialized human resources, impact the use of the method in developing countries (USEPA, 2012; Maciel and Sabogal-Paz, 2016).

In Brazil, the membrane filtration technique is the most used alternative for the research analysis of *Giardia* spp. cysts in various water matrices (Rosado-García *et al.*, 2017), being detected in raw and treated water from rivers (Scherer *et al.*, 2022; Bonatti *et al.*, 2023), treated sewage (Miglioli *et al.*, 2017), marine and estuarine waters (Leal *et al.*, 2018), and swimming pools (Pineda *et al.*, 2020). However, this technique also presents limitations when used in high-turbidity water samples (Coelho *et al.*, 2017).

Calcium carbonate flocculation is an alternative technique that can be applied in high-turbidity water samples but has limited efficacy in samples with low turbidity, i.e., ≤ 1 uT (Andreoli and Sabogal-Paz, 2017). Considering that different methods used to detect pathogenic protozoa in environmental samples have several limitations, often resulting in low recovery efficiency rates, it is critical to encourage research for the improvement of existing techniques as well as developing new strategies that have greater sensitivity for detecting protozoan cysts in high-turbidity water samples.

Filter backwash water (FBW) is a complex matrix that exhibits high turbidity, which would be considered a relevant target for environmental analysis in WTPs since encysted protozoa may accumulate in high concentrations (Petris *et al.*, 2019). Furthermore, this type of sample in some WTPs returns to the beginning of the treatment process or is disposed of at catchment sites or springs, thus reintroducing protozoa into the water treatment systems.

The development of methods that effectively treat FBW or strategies that increase the efficiency of removing these organisms from the filter of WTPs may improve environmental and public health.

This study provides the first evaluation of the recovery efficiency of *G. duodenalis* cysts using an acrylamide-based anionic flocculation polymer (AFP) in filter backwash water samples from a water treatment plant located in Blumenau, southern Brazil and compares it with the calcium carbonate flocculation technique (CCF).

2. MATERIALS AND METHODS

2.1. Experimental design

Over six months, four samples of filter backwash water (FBW) were collected monthly from a water treatment plant in Blumenau, totaling 24 samples. The samples were stored in 1 L glass vials previously washed with Tween 80 (0.01%) until use in the experiments of artificial contamination and recovery of *G. duodenalis* cysts by the calcium carbonate flocculation (CCF) technique (Vesey *et al.*, 1993; Greinert *et al.*, 2004) and with acrylamide-based anionic flocculant polymer (AFP).

The polymer used in the present study (PROFLOC A1000 Series) was kindly donated by Projesan Water & Co. (Gaspar, Santa Catarina, Brazil). It is a high-molecular-weight anionic polymer powder with different loads, developed to enhance the performance of water treatment stations, effluents, and other potential applications.

During the cyst recovery procedure, turbidity values were determined before and after the experiments. Turbidity determination was conducted using a portable turbidimeter model (2100P HACH) via the nephelometric method.

2.2. Inoculum Preparation

To obtain the cysts of the protozoan for the control trial experiments, 30 fecal samples were collected from Howler monkeys kept in captivity in the city of Indaial, Santa Catarina, sourced from the Bugio Project (Bugio, 2018). These samples were processed by centrifuge-flotation in a saturated zinc sulfate solution (Faust *et al.*, 1939), and positive samples were purified by the sucrose gradient concentration method using three densities (Arrowood and Sterling, 1987).

After purification, a direct immunofluorescence assay was employed for the enumeration of cysts and to obtain their average using the Merifluor® kit, following the manufacturer's instructions. The procedures were performed in triplicate with an aliquot of 10 μL of the purified product. Approximately 1000 cysts of *G. duodenalis* were enumerated per mL in the purified product.

2.3. Artificial Contamination Tests

Monthly, two samples of 1 L (FBW) were used for the recovery tests of *G. duodenalis* cysts using the calcium carbonate flocculation (CCF) technique (Vesey *et al.*, 1993; Greinert *et al.*, 2004) and the protocol developed with the use of the acrylamide-based anionic flocculant polymer (AFP). In each of these samples, 1000 cysts of *G. duodenalis* were inoculated.

For each recovery test, a blank test was performed, with the same sample being analyzed by CCF and AFP without the inoculation of *G. duodenalis* cysts. Cysts found in blank tests were discounted compared to those found in recovery tests.

2.4. Concentration tests of *G. duodenalis* cysts:

After adding the inoculum to the two samples, the flasks were left to rest for one hour so that solids with higher densities precipitated and the sample became more clarified.

To evaluate the recovery of *G. duodenalis* cysts by CCF, the supernatant was carefully transferred to another flask that had been previously washed with Tween 80 (0.01%) according to the protocol described by Vesey *et al.* (1993) and Greinert *et al.* (2004).

Briefly, the technique involved adding aqueous solutions of 1M sodium bicarbonate ($NaHCO_3$) and 1M calcium chloride ($CaCl_2$). After shaking, the pH was adjusted to 10 with the addition of sodium hydroxide (NaOH). Following the formation of calcium carbonate ($CaCO_3$) flakes, all samples were left undisturbed for 12 hours to allow protozoa to settle at the bottom of the flasks.

The supernatants were carefully aspirated using a vacuum pump until a volume of 100 mL was obtained, and the precipitate was dissolved in 200 mL of 10% sulfamic acid under manual agitation for 15 seconds. Then, successive centrifugations were performed at 3000× *g* for 10 minutes to obtain a pellet.

To evaluate the recovery of *G. duodenalis* cysts with AFP, the supernatant liquid was transferred to 1 L beakers washed with a Tween 80 (0.01%) solution. Then, 400 µL of anionic polymer (PROFLOC A®) at a concentration of 0.01% was added and subjected to agitation in a jar-test. Rapid stirring at a speed of 400 rpm for one minute was performed to induce coagulation, resulting in a velocity gradient (G) of 365 s^{-1}. Subsequently, flocculation was achieved by reducing the stirring speed to 60 rpm for 20 minutes, resulting in a velocity gradient (G) of 40 s^{-1} (Lin *et al.*, 2023).

Afterward, the sample was transferred to a 1 L bottle and allowed to settle for 12 hours. Following this, the supernatant liquid was discarded by suction using a vacuum pump, and 200 mL of 10% sulfamic acid was added and stirred for 15 seconds to dissolve the flakes formed by the polymer. The solution was then subjected to centrifugation several times at 3000× *g* for 10 minutes until a pellet was obtained.

Certain procedures, such as those described in the calcium carbonate flocculation technique, were retained, including the customary use of 10% sulfamic acid for the dissolution of the flakes, as well as maintaining the centrifuge rotation speed (Figure 1).

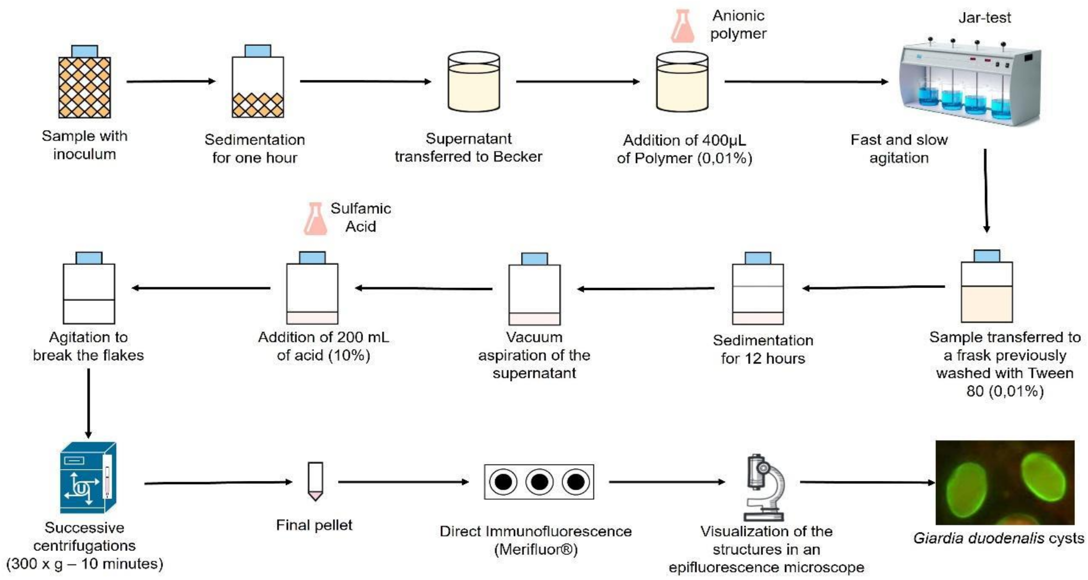

Figure 1. Flowchart with anionic polymer for the detection of *G. duodenalis* in a water sample with high turbidity.

The average number of recovered cysts per liter (L^{-1}) was estimated using the equation described by Franco *et al.* (2001) (Equation 1).

$$X = \frac{N}{K} \times \frac{S}{A} \tag{1}$$

Where:

X: concentration of cysts L^{-1}.

N: number of cysts visualized on the slide.

K: volume of sediment analyzed (µL).

S: volume of the sediment obtained (µL).

A: filtered sample volume (L).

After calculating the cyst estimate, the recovery efficiency (Equation 2) and the relative standard deviation (Equation 3) of each method were determined using the following formulas (USEPA, 2012):

$$Y = \frac{N}{M} \times 100 \tag{2}$$

Where:

Y: recovery efficiency (%).

N: number of recovered cysts.

M: number of seeded cysts.

$$RSD = \frac{SD}{A} \times 100 \tag{3}$$

Where:

RSD: relative standard deviation.

SD: standard deviation.

A: average.

2.5. Statistical analysis

The comparison between the two methodologies involved conducting a normality test using the Shapiro-Wilk Test. The groups were compared regarding the quantitative variable using the Mann-Whitney Test, a non-parametric test. Statistical significance was determined with a threshold of $P < 0.05$.

All the data described were analyzed using Microsoft Excel 2016 software.

3. RESULTS AND DISCUSSION

The use of an anionic polymer resulted in a significant decrease in turbidity, with an average reduction of 98.78%. Additionally, the average recovery rate of *G. duodenalis* cysts was 43.33% (Table 1), consistent with the guidelines established by the US Environmental Protection Agency (USEPA, 2012) for protozoan recovery assays in environmental samples.

Table 1. Comparison of *G. duodenalis* cyst recovery between the calcium carbonate flocculation method (CCF) and the protocol utilizing anionic flocculant polymer (AFP), including recovery efficiency, and changes in sample turbidity before and after the procedures.

Recoveries	Raw Sample Turbidity (uT)	Sample turbidity (uT) after CCF Method	Number of recovered cysts CCF Method	Recovery efficiency CCF Method	Sample turbidity (uT) after AFP Method	Number of recovered cysts AFP Method	Recovery efficiency AFP Method
1	96.7	0.52	800	80%	0.48	200	20%
2	69.5	2.11	400	40%	1.69	600	60%
3	232	1.74	200	20%	1.42	800	80%
4	26	1.04	ND	ND	0.79	200	20%
5	277	0.89	200	20%	0.97	400	40%
6	157	1.54	400	40%	0.57	400	40%
Range	26 – 277	0.52 – 2.11	200 – 800	20 - 80%	0.48 – 1.69	200 – 800	20 - 80%
Average±SD	(143.03 ± 97.27)	(1.31±0.59)	(333.33±244.95)	(33.33±0.24)	(0.99±0.48)	(433.33±233.81)	(43.33±0.23)
P				0.7734			

P: P value of the statistical comparison of the recovery efficiency between the two applied methodologies performed with the Mann-Whitney Test (non parametric that compares two independent groups). If $P < 0.05$, then there are significant differences between groups at the 95% significance level.
SD: standard deviation.
ND: Not Determined.

For the calcium carbonate flocculation approach, the average recovery rate was 33.33%, with an average reduction of 98.39% in turbidity observed (Table 1).

Despite the lower turbidity, the AFP method recovered 20% of cysts in assay number four, while the CCF method yielded no results.

The low turbidity of the sample is a recognized limitation of the CCF technique. Cantusio Neto (2008) evaluated how turbidity influenced the recovery of this method and found that for natural samples with turbidity values less than 50 NTU, the recovery rate for *Giardia* spp. cysts was only 26.5%. Conversely, samples with turbidity values greater than 50 NTU exhibited a better recovery rate of 60.5%.

Franco *et al.* (2012) also assessed the recovery of *Giardia* spp. cysts from reagent water samples, free from interference by other particles, using a commercially standard suspension of cysts. Only one assay using the CCF technique resulted in cyst recovery.

In the same study, the method was applied to recover cysts from samples of raw surface water with high turbidity from Córrego Ressaca, located in Belo Horizonte (MG), and Baixo Cotia, in the metropolitan region of São Paulo (SP). The study found that the average cyst recovery was 39.9% in Minas Gerais and 23.8% in São Paulo.

Greinert *et al.* (2004) conducted a study to evaluate the recovery rate of *Giardia* spp. cysts from highly turbid pool filter backwash water and reported an average recovery efficiency of 18.67% with a standard deviation of ±2.08.

Studies in Brazil utilizing the CCF approach combined with immunomagnetic separation (IMS) have shown slightly improved recovery rates. Giglio and Sabogal-Paz (2018) assessed sludge generated from a water treatment plant and reported an average recovery of 46.1% for *Giardia* spp. cysts. Feng *et al.* (2011) found that the recovery efficiency for *Giardia* spp. cysts ranged from 62% to 66% in China.

Silva and Sabogal-Paz (2020) utilized the detection method on a bench scale with a sample of filter backwash water having a turbidity of 26.7 NTU. The method involved direct centrifugation (DC) with the addition of 1.0% ICN 7X cleaning solution and the use of IMS, resulting in an average recovery rate of 43.9% for *Giardia* spp. cysts

The utilization of the CCF technique may lead to false-positive or negative results due to chemical precipitation occurring at a high pH value over an extended period. The dissolution of calcium carbonate by sulfamic acid can interfere with cyst morphology, rendering it challenging to identify the parasites or altering their viability in infectivity assays. Additionally, the acid can induce a slight increase in fluorescence during microscopic examination (Maciel and Sabogal-Paz, 2016).

This methodology is commonly employed by environmental analysis laboratories because of its cost-effectiveness and ease of implementation. Indeed, it has been utilized for the identification of other protozoa, such as *Toxoplasma gondii* (Silva and Canal, 2021), despite its notable limitations.

Additionally, this technique demonstrates superior efficiency when applied to samples with high turbidity compared to other currently available procedures.

Although ferric sulfate is widely used as a flocculant agent in water treatment plants, it can result in low recovery rates for *Giardia* cysts in high-turbidity water samples like FBW (Silva and Sabogal-Paz, 2021).

In this context, our results demonstrate the practical applicability of using a polymer for the recovery of *G. duodenalis* cysts from complex water samples. We observed an average recovery rate higher than that of the CCF technique, although no significant statistical differences were observed between both methods.

Anionic flocculant polymers, characterized by their negative charges, are typically employed as secondary coagulants in water purification processes. They function by aggregating suspended particles in water, forming them into flakes, and thereby facilitating their separation from the liquid. This method serves as an efficient alternative to conventional coagulants utilized in water treatment, which often comprise metallic salts such as aluminum sulfate, ferric sulfate, and ferric chloride (Criado *et al.*, 2020; Nath *et al.*, 2021).

However, there are relatively few studies that have evaluated the potential of polymers to enhance water quality before use or disposal, particularly in terms of removing protozoan cysts of significant public health concern, such as *Giardia* spp. (Soros *et al.*, 2019; Buenaño *et al.*, 2019).

Several studies have explored the use of plant biocoagulants as a sustainable approach to reduce turbidity in water samples. However, their impact on reducing waterborne pathogens has not yet been thoroughly evaluated. Hadadi *et al.* (2022) conducted a comparative study on the efficiency of seeds from *Opuntia ficus indica*, *Moringa oleifera*, *Aloe vera*, and *Pinus halepensis*, in comparison to two commonly used inorganic coagulants—ferric chloride and alum. The four biocoagulants demonstrated excellent efficiency, removing approximately 100% of the turbidity from the samples.

Other studies have assessed the effectiveness of *Moringa oleifera*, which achieved between 76% and 90% efficiency in reducing turbidity while inducing minimal changes in the electrical conductivity and salinity of water (Bazzo *et al.*, 2022; Rasheed *et al.*, 2023).

Finally, it is important to emphasize that isolating protozoan cysts from complex water matrices presents a significant challenge in environmental parasitology, especially in developing countries where advanced purification procedures like IMS are cost-prohibitive. In this regard, the utilization of alternative strategies for protozoa concentration that are economically feasible enhances the attractiveness of this practice.

4. CONCLUSION

The utilization of anionic flocculating polymer as a practical approach for concentrating protozoan cysts from environmental samples with varying degrees of turbidity has been demonstrated. Therefore, integrating the studied anionic polymer into existing water treatment processes holds significant potential for enhancing both environmental monitoring practices and public health protection activities.

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