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Enhancing *Cryptosporidium parvum* recovery rates for improved water monitoring

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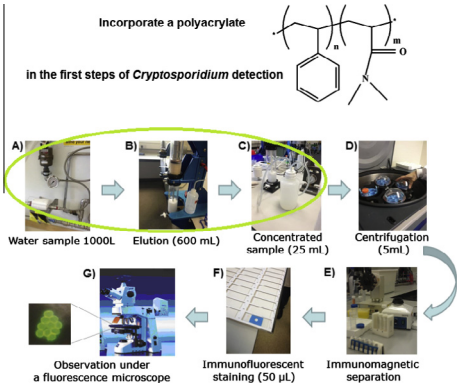
HIGHLIGHTS

- Proposed modified detection method of *Cryptosporidium* with improved recovery rates.
- By using polymer coated filters more oocysts were detected compared to standard.
- Polyacrylates were incorporated in the detection method of *Cryptosporidium*.
- The elution buffer had also an effect on the recovery rates of the filters.

GRAPHICAL ABSTRACT



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ABSTRACT

Water monitoring is essential to ensure safe drinking water for consumers. However existing methods have several drawbacks, particularly with regard to the poor recovery of *Cryptosporidium* due to the inability to efficiently elute *Cryptosporidium* oocysts during the established detection process used by water utilities. Thus the development of new inexpensive materials that could be incorporated into the concentration and release stage that would control *Cryptosporidium* oocysts adhesion would be beneficial. Here we describe improved filter performance following dip-coating of the filters with a "bioactive" polyacrylate. Specifically 69% more oocysts were eluted from the filter which had been coated with a polymer than on the naked filter alone.

1. Introduction

Cryptosporidium is a protozoan parasite which is a major cause of gastroenteritis outbreaks worldwide. In 2010 a waterborne cryptosporidiosis outbreak took place in Sweden and affected at least 27 000 inhabitants of Östersund (Widerström et al., 2014). In the Milwaukee outbreak in 1993 403 000 residents became ill (Corso et al., 2003). Studies revealed that among the residents of Milwaukee, and its vicinity, cryptosporidiosis-associated death increased after the waterborne outbreak (Hoxie et al., 1997). Additionally, medical costs and lost productivity exceeded $96 million (Corso et al., 2003). Waterborne cryptosporidiosis infections have also been detected in Spain (Fuentes et al., 2014), Australia

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(Ng-Hublin et al., 2014), Ireland (Pelly et al., 2007; Glaberman et al., 2002) and France (Dalle et al., 2003). Overall, during the period 2004–2010, 60% of worldwide waterborne parasitic protozoan outbreaks were caused by *Cryptosporidium* spp. (Baldursson and Karanis, 2011).

Water sources in the developed countries can be contaminated by animals, sewage discharge and distribution system failures (Barrett, 2014; Ercumen et al., 2014). *Cryptosporidium* has a very low infectious dose compared with other waterborne pathogens, which can be as low as 10 oocysts (King and Monis, 2007; Okhuysen et al., 1999). The symptoms of *Cryptosporidium* infection depend on the nutritional and immune status of the host and the site of infection which is mainly the small intestine although sometimes the infection may be spread throughout the gastrointestinal tract and extra-intestinal sites (Chalmers and Davies, 2010). Generally the symptoms are diarrhea and abdominal pain nausea, vomiting and low-grade fever, while they are accompanied occasionally with nonspecific symptoms such as myalgia, weakness, malaise, headache, and anorexia (Bouzid et al., 2013). Furthermore cryptosporidiosis can be fatal for children (Huang et al., 2004) and immunocompromised adults. Particularly people with low CD4 counts are at increased risk (Sorvillo et al., 1998, 1994; Hunter and Nichols, 2002). Furthermore there is no fully effective treatment or vaccine for the groups that are vulnerable to the life-threatening cryptosporidiosis while treatments exist for other waterborne pathogens such as *Shigella*, *Escherichia coli* (Striepen, 2013) and *Vibrio cholerae* (Leibovici-Weissman et al., 2014). The increased number of cryptosporidiosis outbreaks worldwide are attributed to *Cryptorporidium* resistance to standard chlorine disinfection (Campbell et al., 1982) and other disinfectants commonly used in laboratories and hospitals (Tzipori, 1983). Furthermore according to the World Health Organization *Cryptosporidium* is among the parasites of primary concern in order to supply safe water to consumers. For the above mentioned reasons regular monitoring of the water supply for the presence of *Cryptosporidium* is undertaken by water utilities. French authorities require the monitoring of *Cryptosporidium* oocysts in drinking waters and generally in water supplies (NF T90-455, AFNOR, 2001; Mons et al., 2009). In 1996, the United States Environmental Protection Agency (USEPA) started the process of acquisition and application of the newest technologies for the detection and identification of *Cryptosporidium* and *Giardia* (U.S. Environmental Protection Agency, 2001). These USEPA1622 and 1623 methods were prepared to give an estimation of the health risk caused by drinking water polluted with the protozoans such as *Cryptosporidium* and *Giardia* (Skotarczak, 2009).

The most commonly used method for the detection of *Cryptosporidium parvum* are the USEPA 1623 (DiGiorgio et al., 2002; Francy et al., 2004; Ongerth and Saaed, 2013) and since 2012 1623.1 (U.S. Environmental Protection Agency, 2012), and ISO 15553 "Water Quality – isolation and identification of *Cryptosporidium* oocysts and *Giardia* cysts from water". In the UK the procedure is similar to this method, starting from 1000 L of water with UK companies using the IDEXX Filta-Max filters. Due to the pore size of the cartridge filter all the *Cryptosporidium* oocysts are captured. Afterwards the oocysts are eluted from the IDEXX Filta-Max filter and concentrated on top of a membrane. Then the oocysts are eluted from the membrane, further concentrated by centrifugation and purified by immunomagnetic separation. Finally the oocysts are dissociated from the immunomagnetic beads, stained and enumerated under a fluorescence microscope (Fig. 1).

However current detection procedures, e.g. USEPA 1623, have less than 100% recovery rates. Recovery rate is the efficiency of the process in concentrating all of the pathogens of interest. For example a recovery rate of 100% means that all the pathogens of interest in the original sample are detected after the process. Recovery is particularly important for pathogens present in low numbers, as if the rate is low any pathogen in the sample might be lost during the sample processing stage. This is assumed to be due to the inability of controlling the elution of *Cryptosporidium* oocysts during the concentration stage of the process, with a wide range of average recovery efficiencies reported in the concentration stage, with one study finding average values of about 22%, while the average recovery rates for the next stages are above 60% (Rony, 2010). However 38% recovery is considered acceptable within the USEPA method 1623.1 guidelines (U.S. Environmental Protection Agency, 2012). According to Hu et al. (2004) the recovery rate of *C. parvum* oocysts was 18% when including the filtration step. Taking into account the infectious dose of *Cryptosporidium* it is obvious that the achieved recovery rates are low. Therefore modification of the concentration stage will lead to more accurate results. Thus modifications to USEPA 1623 have been proposed in the literature (Kimble et al., 2013). The recovery rates are impacted by the type of filter, the elution buffer, the water type and operator skill (Hill et al., 2005; Holowecky et al., 2009; Polaczyk et al., 2008). The recovery efficiency of the filter is influenced by its material but comparisons in water applications of different materials are difficult as the filter design itself varies.

Here we provide the first investigation of how the material influences the recovery rate of *Cryptosporidium* filters, using coated commercial filters in order to compare the performance of two different materials. An ideal material would have very good stability and long term durability. They will not be toxic or irritating to people who are handling/using them and they will be insoluble in water. In this study it is shown that the elution/recovery of *C. parvum* oocysts is enhanced by using commercially available filters which have been dip-coated with a specific polyacrylate that prevents *Cryptosporidium* adhesion, the polymer having been identified from a recent study (Wu et al., 2012).

2. Materials and methods

2.1. Materials

The foam discs and the membrane filters were obtained from IDEXX. The polymers PA6 and PA531 were synthesized via radical polymerization on a mmol scale as previously reported (Wu et al., 2012; Patent WO 2013/079938). The solvents tetrahydrofuran, toluene, hexane, diethyl ether, acetone and acetic acid were all obtained from Sigma–Aldrich. Crypto-a-Glo™ was obtained from Waterborne Inc. Oocysts were obtained from Scottish Water via Creative Science Company (oocysts were the Moredun isolate) and enumerated using FACS.

2.2. Filter preparation

Solvent Selection: Initially the goal was to find a solvent for the polymers that would not dissolve the filters. Thus commercially available filters were immersed for 5 min into three different potential coating solvents specifically tetrahydrofuran, acetone and acetic acid and the weight of each filter recorded before and after immersion.

Filter Coating: From the above experiment tetrahydrofuran was selected as the best solvent. Thus the filters were coated by dip-coating for 5 min into the polymer dissolved in tetrahydrofuran at concentrations of up to 1% (w/v). After the dip-coating the filters were dried in a fume hood for 24 h and the weight recorded to give coating efficiencies. The polymer loading is the percentage increase in the weight in comparison to the initial weight.

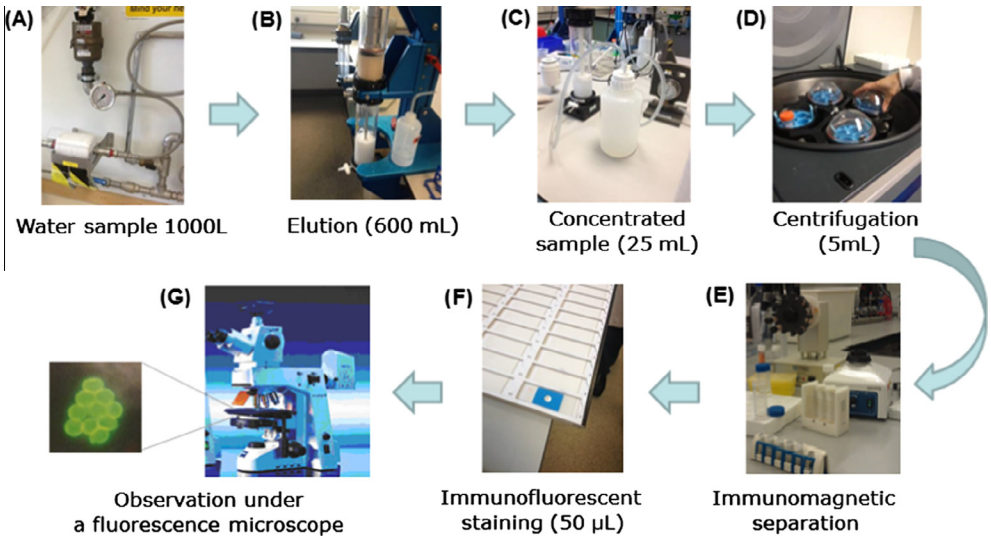

Fig. 1. Protocol of USEPA 1623.1 for *Cryptosporidium* detection process by using IDEXX Filta-Max filters.

2.3. Recovery rate tests with parasites

2.3.1. Measure the recovery rates of the coated membranes

For evaluating the performance of the coated membranes 100 *C. parvum* oocysts in 5 mL water were pipetted on top of the coated and uncoated membranes and pressure applied (approximately 40 bar). Then the membranes were placed into a membrane bag and eluted using firstly 25 mL, followed by another 20 mL, of PBS (membranes were rubbed between finger and thumb for 1 min as in the USEPA 1623 method). Finally the membrane bag was rinsed with 5 mL of PBS.

The membrane elutes were centrifuged at 1100 g for 15 min, the supernatant was discarded and 5 mL (pellet) directly transferred to a Dynal Leighton tube with 5 mL of water added and vortexed. To each tube 1 mL of Dynal SL-Buffer A and 1 mL of SL-Buffer B and anti-*Cryptosporidium* Dynabeads (100 µL) were added. Tubes were placed on the Dynal sample rotor at 15–25 rpm. After 1 h, the Leighton tubes were placed in a magnetic particle concentrator (MPC-1), with the flat side of the tube towards the magnet. The MPC-1 was rotated through 90° for a minimum of two minutes, with approximately one tilt per second. The supernatant was discarded and the Leighton tubes were removed from the MPC-1. To each tube 900 µL of 1×SL A buffer was added to wash the contents of the Leighton Tube paying attention to the bottom and flat side of the tube and was transferred using a Pasteur pipette to an appropriately labelled microcentrifuge tube.

A further 100 µL of 1×SL A buffer was then added for a wash, and this was then transferred into the same microcentrifuge tube as before using the same Pasteur pipette to avoid losses. The samples were placed in the MPC-S, with the magnetic strip in the upright position. The MPC-S was rotated through 180° for a minimum of 2 min. With the microcentrifuge tubes still in the MPC-S, the supernatant was removed using a Micropipette and discarded. The magnetic strip was removed from the MPC-S and 50 µL of 0.1 N Hydrochloric acid (to dissociate bead-pathogen complexes) was added to each tube and vortexed for 10 s. The tubes were left to stand in a vertical position for 5 min then vortexed for another 10 s.

The magnetic strip was placed into the MPC-S in the angled position and left for approximately 10 s to remove the magnetic beads. Then 5 µL of 1 N Sodium Hydroxide was added to well of a set of microscope slides. The samples from the microcentrifuge tubes were transferred onto the prepared slides. The slides were placed at room temperature overnight to dry completely. Methanol (50 µL) was added to each slide and air dried at room temperature. Cellabs Cryptocel FITC reagent (50 µL) was added to each slide and incubated at 37 °C for 15 min. The FITC reagent was then removed using a pipette, 50 µL of a 1:5000 DAPI solution was added and the slides were incubated at 37 °C for 5 min. This solution was then removed using a pipette and 50 µL of ultrapure water was added as a wash. This was left for 1 min and then removed using a pipette. 10 µL Cellabs Mounting fluid was added and mounted with a cover slip. At the end the slides were sealed using clear nail varnish, allowed to dry, and the *Cryptosporidium* oocysts were enumerated at Moredun Scientific using a microscope.

2.3.2. Measure the recovery rates of the coated foam discs

For evaluating the coated sponges a small scale experiment was used in order to evaluate the performance of the coated filters regarding the capturing and the elution of *C. parvum* oocysts. 100 *C. parvum* oocysts in 1.5 mL DI water were pipetted on top of coated and uncoated filters and left for 3 h. Afterwards elution of the filters with phosphate buffered saline (PBS) and PBS with 0.01% Tween (PBST) was performed in order to evaluate the "release" of the *C. parvum* oocysts. The staining of the *C. parvum* oocysts was performed by following the standard *C. parvum* staining protocol (EPA1623).

The liquid samples were passed through an isopore membrane. Then the *Cryptosporidium* oocysts that were on top of the membrane were stained with Crypto-a-Glo™. Then the samples were incubated in a humid chamber at 37 °C for 25 min. A 10 µL of M101 Mounting fluid was added and mounted with a cover slip. At the end slides were sealed using clear nail varnish and the *Cryptosporidium* oocysts were enumerated at Scottish Water.

3. Results and discussion

Two approaches were followed in order to improve the recovery rate of the established method presented in Fig. 1. The first approach was the dip-coating of the membrane and the second dip-coating of the foam discs. Particularly, in the first approach the step C (Fig. 1) was modified by changing the membrane properties through dip-coating with polymers whereas in the second

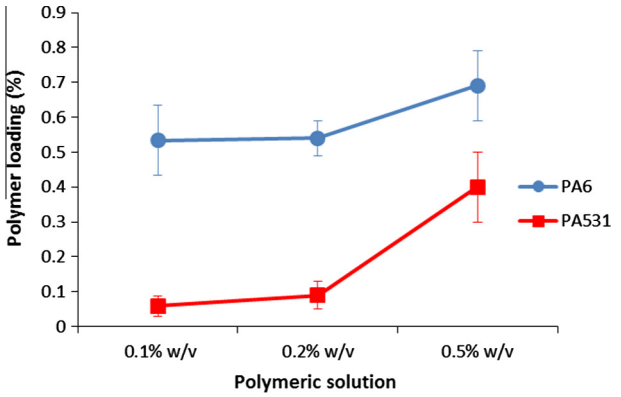

Fig. 2. Polymer loading after dip-coating of membranes into different polymeric solutions of PA6 (blue circles) or PA531 (red squares). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

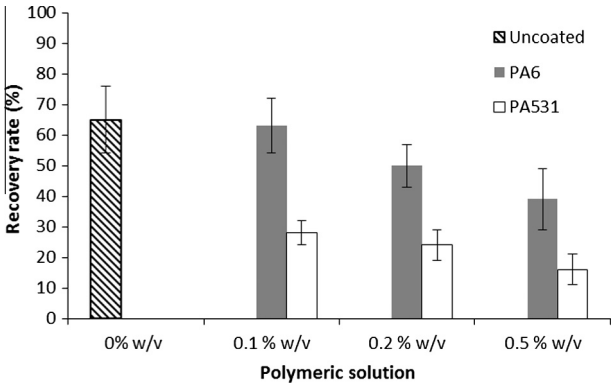

Fig. 4. Recovery rates obtained from uncoated membranes (hatched column), membranes dip-coated into different polymeric solutions of PA6 (grey columns) and PA531 (white columns). Each point is the mean value of three measurements ± SD obtained by three replicates.

approach steps A and B (Fig. 1) were modified by changing the foam discs properties through dip-coating of the foam discs.

3.1. Dip-coating of the membranes

3.1.1. Membrane solubility

Initially membrane solubility experiments were performed. For this purpose the weight of the membranes was recorded before and after immersion into the solvents for 5 min. The membranes were taken out from the solvents and dried for 24 h and the weight recorded. It was observed that the commercially available membranes were partially dissolved in acetone, while acetic acid remained in the membranes even after 72 h. On the other hand the solubility of the membranes by tetrahydrofuran was negligible. Particularly the weight increased only 0.01% upon immersion of the membranes in tetrahydrofuran (Supplementary material Table S1). Thus tetrahydrofuran was selected as the appropriate solvent for the polymers.

3.1.2. Polymer selection

Two different polyacrylate polymers were used to coat commercially available filters and investigate the impact upon their recovery rates. The first selected polymer was PA531 that enhances the adhesion of *C. parvum* oocysts and is expected that this polymer will trap the oocysts on the foam discs and membranes and thus to decrease the recovery rates. PA531 is composed of MEMA (Methoxyethyl methacrylate) and DEAEMA (2-(Diethylamino) ethyl methacrylate). The second selected polymer was PA6 that prevented their adhesion and is expected to help the elution of the oocysts and thus to improve the recovery rates. PA6 is composed of styrene and DMAA (N,N-Dimethyl acrylamide) (Supplementary material Fig. S1).

3.1.3. Membrane coatings

The filters were coated with the polymers by dip-coating into the polymeric solutions. Three replicates were fabricated for each different polymeric solution. The results are presented in Fig. 2.

As it is shown in Fig. 2, dip-coating of the membranes into different polymeric solutions resulted into different polymer loadings. Specifically for both PA6 and PA531 increasing the polymer concentration resulted in increased polymer loading to the membranes upon dip-coating.

3.1.4. Impact of the polymer loading on pore size and the flow rate of the membranes

Although the highest polymer loading would improve the performance of the membranes regarding the elution of *Cryptosporidium* oocysts the impact on the flow rates of the membranes is of great importance to water monitoring processes. Thus the pores of the membranes upon dip-coating accomplished were observed through SEM measurements in order to observe on the one hand the retention of pore size and on the other hand the clogging of the membranes. For that purpose samples of the coated membranes were compared with the uncoated ones (Fig. 3).

As it is demonstrated in Fig. 3 the membranes dip-coated into 1% w/v polymeric solutions did not retain their pore sizes either upon dip-coating in PA6 or PA531. Furthermore, flow rate

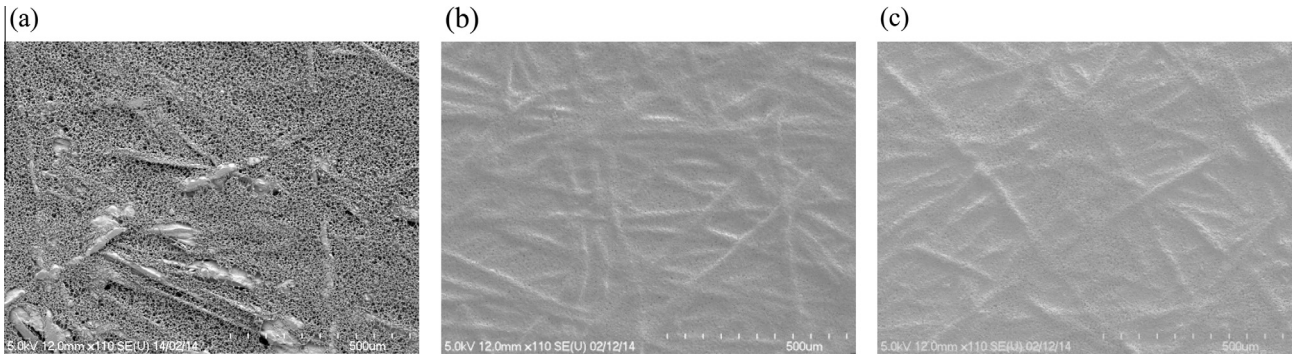

Fig. 3. SEM images of (a) uncoated membrane, (b) PA6 coated membrane, (c) PA531 coated membrane. The coated membranes were prepared upon dip-coating into 1% w/v polymeric solutions for both polymers.

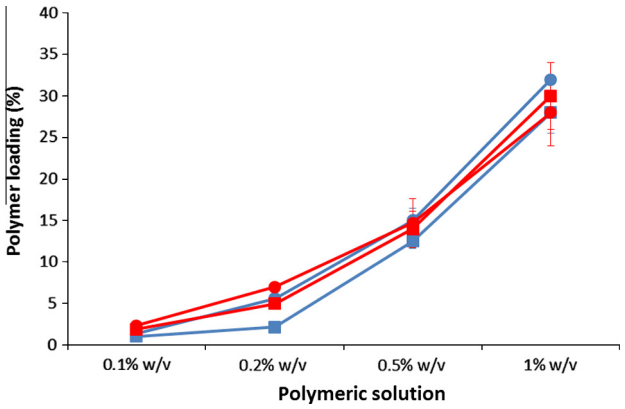

Fig. 5. Polymer loading versus dip-coating into different polymeric solutions obtained from large foam discs (circles) and small foam discs (squares) after dip-coating in different polymeric solution of PA6 (blue lines) or PA531 (red lines). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

measurements were performed as well to fully characterize the coated membranes.

By comparing the flow rates of the coated membranes (average values presented in Supplementary material Table S2) with the flow rates of the uncoated membranes, it is concluded that many of the pores were blocked or narrowed after dip-coating even at 0.1% w/v with the flow rate decreasing as the polymer loading increases as expected.

3.1.5. Performance of the coated membranes

Following characterization of the coated membranes, the recovery rates regarding the elution of *Cryptosporidium* oocysts were measured following the procedure presented in Section 2.3.1.

As demonstrated in Fig. 4 the recovery rates are higher for PA6 than for PA531 as per our hypothesis. However, recovery rates decrease as the polymer loading increases and only the lowest PA6 concentration is comparable to the uncoated case. The explanation for this trend is the impact of flow rate upon performance. Particularly the lower recovery rates in comparison with the uncoated ones are attributed to the trapping of the *Cryptosporidium* oocysts inside the membranes due to the clogging of the membranes causing also a resulting difficulty of elution during the next step.

3.2. Dip-coating of the foam discs

3.2.1. Foam disc solubility

The second approach of the method was followed in order to improve the recovery rates. As mentioned above the initial study was the selection of the appropriate solvent for the polymers with respect to the filter solubility. For that purpose again the weight of the foam discs was recorded before and after immersion into the solvents for 5 min. It was observed that the commercially available foam discs were partially dissolved in acetone, while acetic acid remained again in the filters even after 72 h drying. However the solubility of the foam discs in tetrahydrofuran was the lowest. Particularly the weight of the small foam disc decreased only 0.37% compared to its initial weight, while the weight of the large foam disc decreased only 0.19% compared again to its initial weight (Supplementary material Table S3). Thus again tetrahydrofuran was selected as the appropriate solvent for the polymer coating of the foam discs.

3.2.2. Foam disc coating

The foam discs were coated with the polymers by dip-coating into the polymeric solutions. The polymer loading was calculated by three replicates and the results are presented in Fig. 5. Dip-coating of the foam discs into different polymeric solutions resulted into different polymer loadings as expected (Fig. 5).

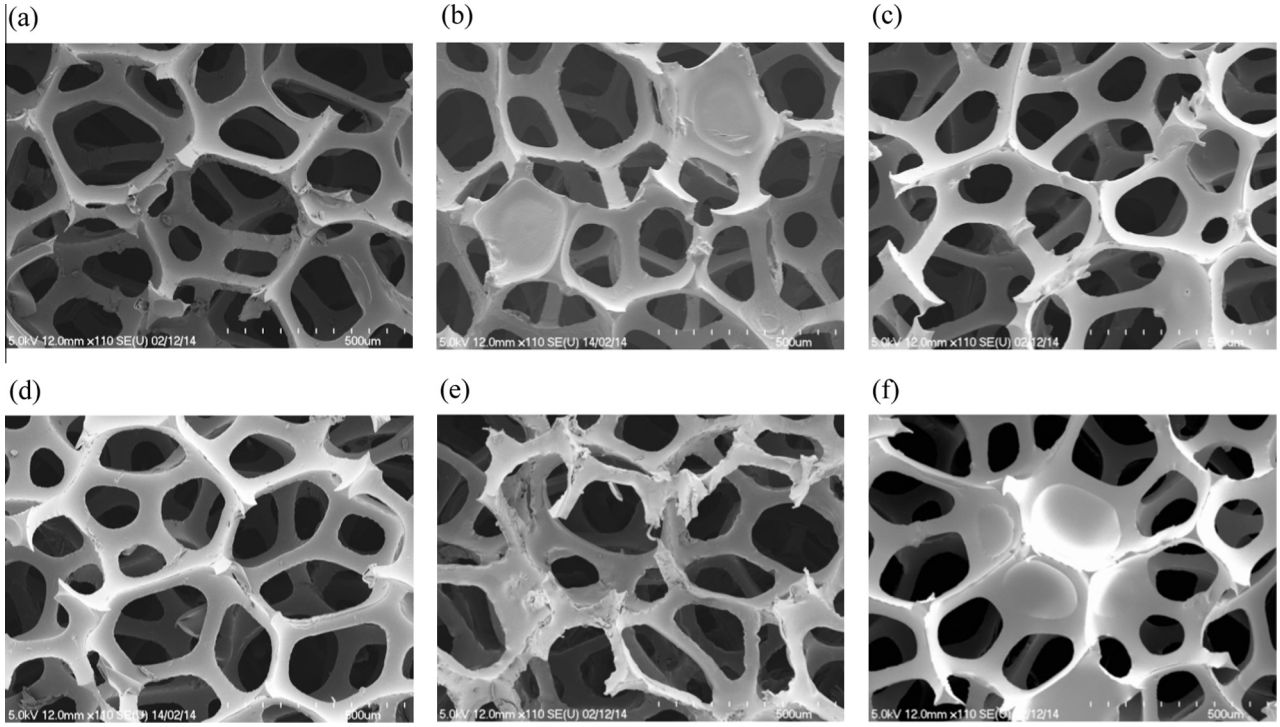

Fig. 6. SEM images of (a) uncoated small foam disc, (b) PA6 coated small foam disc, (c) PA531 coated small foam disc, (d) uncoated large foam disc, (e) PA6 coated large foam disc, (f) PA531 coated large foam disc.

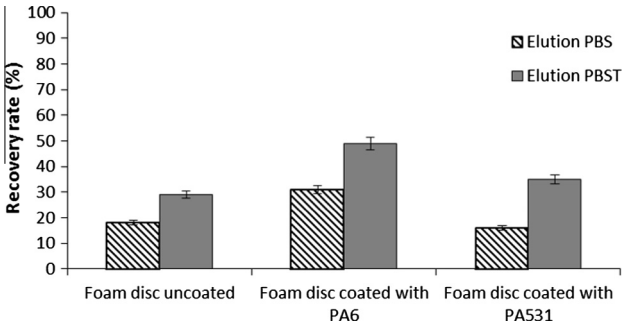

Fig. 7. Recovery rates obtained from uncoated foam discs, coated foam disc with PA6 and PA531 after dip-coating into 1% w/v polymeric solution for elution in PBS (hatched columns) and elution in PBST (grey columns). Each point is the mean value of three measurements ± SD obtained by three replicates.

3.2.3. Verification of polymer loading and pore size retention

The distribution of the pore sizes of the coated foam discs upon dip-coating in 1% w/v polymeric solution were compared with uncoated foam discs through SEM measurements (Fig. 6). Herein it was observed that the coated filters had few clogged pores (Fig. 6) while the polymer loading was also verified.

3.2.4. Performance of the coated foam discs

The performances of the coated foam discs regarding the elution of *C. parvum* oocysts were compared with uncoated foam discs. As shown in Fig. 7, it was observed that the most oocysts were eluted from the foam disc coated with the PA6 polymer and the fewer were eluted from the foam disc coated with PA531. Also the elution buffer had a major effect on the recovery rates of the coated foam discs. Particularly for the uncoated and the PA6 coated foam discs PBST improves elution by a factor of approximately 1.5 whereas it doubles for PA531. Notably, the foam discs coated with PA531, performed better than the standard material when the elution was performed in PBST (Fig. 7).

The increase in the recovery rates for PA6 coated foam discs in comparison with uncoated foam discs were statistically significant in both elution conditions (t-test, $p < 0.002$). Overall the PA6 coated foam discs performed better regarding the elution of *Cryptosporidium* oocysts demonstrating 72% improvement in the case of PBS and 69% improvement in the case of PBST, proving that the coating of the foam discs is a good approach to improve the recovery rates of the established method.

4. Conclusions

Cryptosporidiosis can be fatal for immunocompromised adults, the young and the elderly. Until now there is no fully effective treatment for human cryptosporidiosis. Cryptosporidiosis outbreaks are mostly associated with *C. parvum* and *C. hominis*. Both *C. parvum* and *C. hominis* have very low infectious doses and are highly resistant to both environmental stress and standard chlorination disinfection procedures. Taking into account these facts it is clear that the detection of *Cryptosporidium* is crucial for the public health. However the recovery rates of the already established detection methods are considerably low. This is mostly attributed to the inability of controlling the "release" of *Cryptosporidium* oocysts during the multistep detection procedure because on the one hand it is extremely important to trap all the oocysts of the sample into the filter, while on the other hand it is equally important to elute all the oocysts from the filter in order to detect them. Thus the ultimate goal is both the capturing and the "release" of the oocysts to be achieved at 100%.

To push towards this goal two different polyacrylate polymers were chosen in order to coat commercially available foam discs and membranes and to trap *Cryptosporidium* oocysts or prevent their adhesion. The achieved recovery rates of the coated foam discs and membranes were compared with uncoated ones. Overall increased recovery rates were observed for coated foam discs only. Particularly, in the case of the coated foam discs the impact of the flow rate was not considered and we observed a significant increase in recovery rates in non-flow situations. It was observed that 69% more oocysts were eluted from the foam discs coated with the PA6 polymer compared to the uncoated foam discs, and fewer were eluted from the foam discs coated with PA531, when the elution was performed with PBST. Also as demonstrated in our experiments when the elution from the foam discs was performed in PBST more oocysts were eluted from all the samples. Regarding the PA6 coated foam discs they had higher recovery rates for both elution conditions in comparison with the standard uncoated material.

In the case of coated membranes there was no significant improvement regarding recovery rates, while the coating impacts severely on the flow rates of the membranes. Therefore as a future direction it would be promising to explore the fabrication of filters directly from the appropriate adhesion preventing polymeric materials to improve the already established detection method. Also another perspective is the use of membranes with larger pore sizes that can then be coated with polymers in order to shrink their pores and enhance the recovery rates of *Cryptosporidium* oocysts.

Acknowledgements

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chemosphere.2015.05.021.

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