Journal of Water & Health

Check for updates

© 2023 The Authors

Journal of Water and Health Vol 21 No 10, 1580 doi: 10.2166/wh.2023.217

Comparison of four commercial immunomagnetic separation kits for the detection of *Cryptosporidium*

Jeanne Claudeen Arona*, Timothy J. Hall, Flyn Mckinnirey and Fei Deng
Graduate School of BioMedical Engineering, University of New South Wales, Sydney 2052, Australia
*Corresponding author. E-mail: j.arona@unsw.edu.au

ABSTRACT

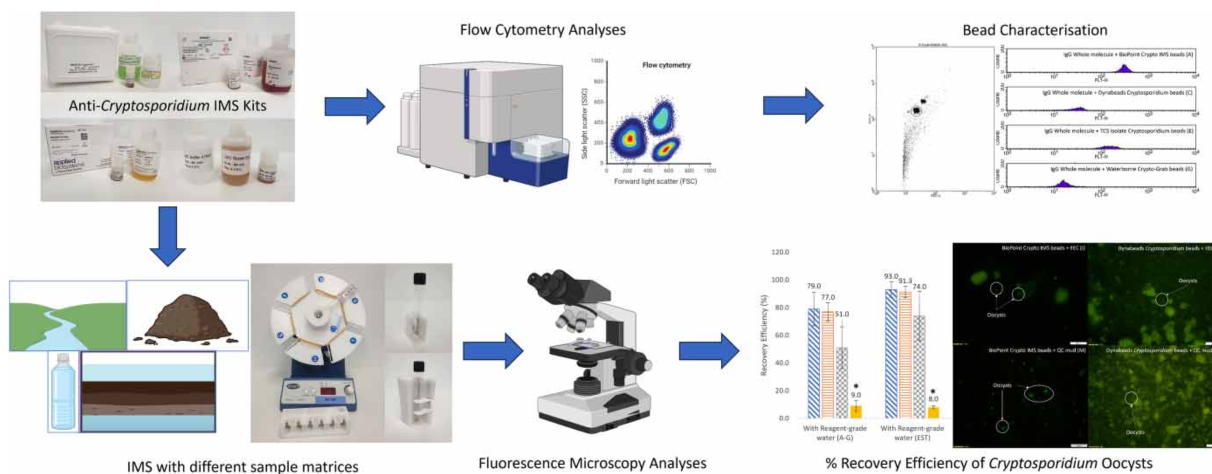
Cryptosporidium spp. are protozoan parasites of significant health importance found in environmental waters globally. Four commercially available *Cryptosporidium*-specific immunomagnetic separation (IMS) kits used in various water sample matrices were analysed and compared. Beads were characterised by flow cytometry and tested for the recovery efficiencies for oocysts spiked into different matrices: river water sediment, clay sample, and filter backwash sample. Results showed that Dynabeads™ *Cryptosporidium* and Waterborne Crypto-Grab™ kits contained immunoglobulin IgM antibody-coated beads. In contrast, the BioPoint CryptoBead and the TCS Isolate kits contained immunoglobulin IgG antibody-coated beads. BioPoint CryptoBead was significantly coated with more antibodies and were able to capture oocysts more rapidly compared to the other beads. Recovery efficiencies of Dynabeads™, TCS Isolate® beads, and BioPoint CryptoBead ranged from 55 to 93% when tested against different sample matrices, with BioPoint CryptoBead resulting in the highest at 93% in reagent-grade water and Dynabeads™ at 55%, the lowest against clay samples. The Waterborne beads did not perform well on any samples, with recovery efficiencies ranging from 0 to 8%. Fluorescence microscopy analyses showed that both the IMS method and the sample matrix processed affect the quality of the membranes, with the cleanest samples for microscopy examination observed from BioPoint CryptoBead.

Key words: antibody, *Cryptosporidium*, immunomagnetic separation (IMS), magnetic beads, oocyst, recovery efficiency

HIGHLIGHTS

- Antibody types on magnetic beads and IMS methods differ across commercially available IMS kits.
- IgG-coated BioPoint beads had 75–93% recovery efficiencies across the different sample matrices and gave the cleanest slides for microscopy.
- IMS recovery efficiency is influenced by both the method used and the matrix analysed, with the purity of the slides affecting the results.

GRAPHICAL ABSTRACT



This is an Open Access article distributed under the terms of the Creative Commons Attribution Licence (CC BY 4.0), which permits copying, adaptation and redistribution, provided the original work is properly cited (http://creativecommons.org/licenses/by/4.0/).

Downloaded from http://iwaponline.com/jwh/article-pdf/21/10/1580/1312367/jwh0211580.pdf
by guest

INTRODUCTION

Enteric protozoa known for their infectivity, *Cryptosporidium* spp., are parasitic pathogens that can be transmitted in humans via the faecal–oral route including from contaminated water sources and food production (Ryan *et al.* 2021; Costa *et al.* 2022). First reported to cause gastrointestinal infection in humans in 1976 (Nime *et al.* 1976), *Cryptosporidium* has now been considered a leading cause of diarrhoeal illnesses (cryptosporidiosis) to both young and immunocompromised individuals globally (O'Leary *et al.* 2021). Approximately 90% of cryptosporidiosis cases in humans are caused by *C. hominis* and *C. parvum* (Hassan *et al.* 2021). *Cryptosporidium* has a low infectious dose, and the environmentally robust oocysts are resistant to chlorine disinfectants, including those used in water treatment plants (Temesgen *et al.* 2021). Additionally, it has been identified that *Cryptosporidium* oocysts are able to survive for months in soil and water and are able to pass through filters for water treatment due to their small size of ~4 to 5 μm (Andreoli & Sabogal-Paz 2019). Monitoring water sources for the prevalence of *Cryptosporidium* and setting up surveillance systems are important to validate the efficiency of water treatment; however, there are challenges with the existing analytical methods.

Currently, the gold standard for the detection of both *Cryptosporidium* and *Giardia* spp. in water is a three-step procedure outlined by the U.S. Environmental Protection Agency in Method 1623.1 (Hassan *et al.* 2021). The three main steps are (1) the concentration of the sample using filtration and centrifugation, (2) the separation of the sample using immunomagnetic separation (IMS), and (3) the enumeration of oocysts using immunofluorescent staining and microscopic analysis.

The IMS procedure involves adding antibody-coated magnetic beads to the concentrated sample and incubating to allow the beads to bind to the oocysts. The process is carried out in borosilicate glass culture tubes called Leighton tubes with flat sides and adjoining magnetic clips. In these Leighton tubes, the IMS buffers are incubated with the magnetic beads and the water sample of interest. The bead–oocyst complexes are magnetically separated from the solution, then the attached oocysts are dissociated from the beads via heat or 0.1 M hydrochloric acid treatment (Andreoli & Sabogal-Paz 2021).

Several different anti-*Cryptosporidium* IMS bead kits are commercially available. These include Dynabeads™ *Cryptosporidium* Kit (IDEXX) ('Dynabeads'), Crypto-Grab™ *Cryptosporidium* oocyst Isolation kit from Waterborne, Inc. ('Waterborne beads'), Isolate® *Cryptosporidium* IMS kit from TCS BioSciences ('TCS beads'), and CryptoBeads *Cryptosporidium* IMS beads from BioPoint Pty. Ltd (Sydney, Australia) ('BioPoint beads').

The aim of this study is to assess and compare the performance of anti-*Cryptosporidium* IMS kits currently available in the market, which are tested against different sample matrices. These will be carried out by analysing the recovery efficiencies of spiked oocysts as well as assessing the purity and ease of reading the slides during microscopy.

METHODS

Materials and reagents

Phosphate-buffered saline (PBS), bovine serum albumin (BSA), anti-Mouse IgG (whole molecule)-fluorescein isothiocyanate (FITC) antibody produced in goat (anti-mouse IgG FITC), and anti-mouse IgM (μ-chain-specific)-FITC antibody produced in goat (anti-mouse IgM FITC) were purchased from Sigma Aldrich. BD TruCount™ Absolute Counting Tubes In Vitro Diagnostic (IVD) were purchased from BD BioSciences. For the IMS kits, CryptoBead *Cryptosporidium* IMS beads were supplied by BioPoint Pty. Ltd, Crypto-Grab™ *Cryptosporidium* oocyst Isolation kits were purchased from Waterborne, Inc., Dynabeads™ *Cryptosporidium* kits were purchased from IDEXX, and Isolate® *Cryptosporidium* IMS kits were purchased from TCS BioSciences.

The magnetic beads were briefly described by their respective manufacturers as follows: BioPoint beads are coated with a purified anti-*Cryptosporidium* oocyst monoclonal antibody; Dynabeads are coated with purified antibodies against *Cryptosporidium* oocysts covalently bound to the surface; TCS beads are conjugated with oocyst wall-specific anti-*Cryptosporidium* mouse monoclonal IgG; and Waterborne beads are coated with the manufacturer's own mouse monoclonal antibodies prepared specifically against antigens located on the outer surface of oocysts. The accuracy of Dynabeads was stated to be determined by seeding experiments and the recovery efficiency might be affected by factors of the water sample concentrate seeded, such as relative turbidity. Apart from these, no additional information was available regarding the specific properties of the beads as well as on cross-reactivity, specificity, and sensitivity of the IMS kits.

Different sample matrices were analysed in IMS. Reagent-grade water was obtained from Livingstone (Sydney, Australia), while Cockle Creek Sediment is a river water sediment collected in Ku-ring-gai National Park, Australia. The clay sample was prepared from Fuller's Earth Clay purchased from N-essentials Pty. Ltd, and the quality control (QC) mud was a filter

Downloaded from http://iwaponline.com/jwh/article-pdf/21/10/1580/1312367/jwh0211580.pdf
by guest

backwash sample from a water treatment plant in Sydney, Australia. Samples from the Cockle Creek Sediment, Fuller's Earth Clay, and QC mud were washed and resuspended in distilled water prior to use. Preliminary screening for the presence of indigenous oocysts was conducted on these sample matrices using the Dynabeads GC-Combo kit and ColorSeed™.

For all recovery experiments conducted, ColorSeed® flow cytometry dispensed control samples were used (BioPoint, Pty. Ltd). These were suspensions that contained precisely enumerated oocysts, i.e., 100 or 1,000, which have been labelled with Texas Red and can be confirmed with fluorescence microscopy (Warnecke *et al.* 2003). ColorSeed Tween Rinse (BioPoint Pty. Ltd) was used to rinse ColorSeed tubes to ensure all oocysts are transferred to the tubes and to prevent the aggregation of oocysts when seeding.

Immunofluorescence staining was carried out using EasyStain™ kits provided by BioPoint Pty. Ltd and the Aqua-Glo™ G/C Direct Comprehensive Kit purchased from Waterborne, Inc.

Flow cytometric analysis on anti-*Cryptosporidium* IMS beads

For each type of magnetic beads, a 2 μL aliquot was suspended in a 100 μL anti-mouse IgG whole molecule or IgM μ-chain-specific FITC prepared to 1:32 working concentration with 1% BSA in PBS, vortexed thoroughly, and incubated for 30 min at room temperature. After staining, 200 μL of 1% BSA in PBS was added and samples were kept on ice until analysis. Flow cytometric acquisition was performed on a BD FACSCalibur and data analyses with BD CellQuest™ (BD BioSciences).

For fluorescence detection, the samples were initially identified using the forward scatter (FSC) and side scatter (SSC) detectors and then FITC fluorescence was measured using the FL-1 detector. TruCount™ tubes were used to determine the number of magnetic particles per millilitre (mL) of bead suspension into each tube. About 995 μL of 1% BSA in PBS and 5 μL of the magnetic particles were added and vortexed thoroughly.

Capture efficiency analyses

The efficiency of the IMS beads to capture oocysts was assessed in three replicates for each kit as follows. In 1.5 mL microcentrifuge tubes, 100 μL of Buffer A (10×) and 100 μL of Buffer B respective for each kit were added. One vial of customised ColorSeed, given at 0.9 mL volumes, with 1,000 *Cryptosporidium* oocysts was vortexed and added to the microcentrifuge tube, and the ColorSeed tube was rinsed with ColorSeed Tween Rinse and the washings were added to the microcentrifuge tube. To each tube, 10 μL of the IMS beads were added. The samples were incubated with gentle rotation for 5, 15, and 30 min. The tubes were then attached to Isolate Micro-clip (ZAICS5, TCS BioSciences) magnetic clips and incubated for another 5 min. With the bead pellets captured on the magnets, the supernatants were pipetted onto membrane filters and stained as indicated below.

Immunomagnetic separation

IMS was initially performed on reagent-grade water samples following the manufacturer's instructions respective to each brand. Similar sample preparation steps were instructed for BioPoint beads, Dynabeads, and TCS beads where 1 mL each of Buffer A (10×) and Buffer B were dispensed into tubes. Waterborne beads required the addition of Grab Buffer A (2×) only. One vial of ColorSeed was added to the tube and rinsed with ColorSeed Tween Rinse. Reagent-grade water was added to the tube, followed by 100 μL of IMS beads. The tubes were incubated at room temperature with gentle rotation as prescribed (a minimum of 60 min for all three kits and a minimum of 15 min for BioPoint beads). The tubes were then attached to magnets for magnetic capture of the bead–oocyst complexes. After tipping off the supernatants, the beads were rinsed out of the tubes and transferred into 1.5 mL microcentrifuge tubes for at least one washing step with Grab Buffer B for Waterborne beads and with the diluted Buffer A (1×) for the other three kits. To dissociate the oocysts from the beads, 50 μL of 0.1 M HCl was added to the microcentrifuge tubes. The tubes were vortex-mixed and then incubated at room temperature for 10 min before magnetic separation. The supernatant was collected and pipetted onto membrane filters as indicated in the Post IMS Analyses section.

IMS was similarly performed on the three sample matrices: Cockle Creek Sediment sample, Fuller's Earth Clay sample, and QC mud sample, each tested at 0.1 mL packed pellet volume. All the experiments were done in three replicates. As a contamination test, negative control assays were performed where the relevant IMS procedure for each respective kit was tested against distilled water without any oocyst spike. All control assays showed negative results for the presence of *Cryptosporidium* oocysts.

Downloaded from http://iwaponline.com/jwh/article-pdf/21/10/1580/1312367/jwh0211580.pdf
by guest

Post IMS analyses

Immunofluorescence analyses were performed using Aqua-Glo™ kits and EasyStain™ kits to stain the oocysts. Isopore polycarbonate (PC) membrane filters of 13 mm diameter (Merck Millipore) with 0.8 μm pore size were placed onto 13 mm Swinnex filter holders set up on a manifold to allow drainage by vacuum pressure. The samples were pipetted onto the membrane and drained by vacuum. The membranes were stained with one drop of the fluorescent antibody for 15 min (EasyStain™) or 25 min (Aqua-Glo™), rinsed with Wash Buffer, and then mounted on glass slides for microscopy analyses.

Membranes were analysed using epifluorescence microscopy with an Olympus® BX51 microscope. FITC staining was detected using a FITC filter (excitation, 480 nm; emission, 520 nm), and a Texas Red filter (excitation, 560 nm; emission, 630 nm) was used to confirm the target oocysts.

Recovery efficiency was calculated using the formula given in Equation (1), where D_1 is the oocysts obtained from the first acid dissociation; D_2 is the oocysts recovered from the second acid dissociation; and ColorSeed (CS) is the number of inoculated oocysts for each replicate.

$$\text{Recovery efficiency} = \frac{D_1 + D_2}{\text{CS}} \times 100\% \tag{1}$$

Equation (1) represents the recovery efficiency formula.

Statistical analyses

The statistical analysis was performed using Microsoft® Excel® for Microsoft 365 MSO (Version 2306 Build 16.0.16529.20100). Significance was determined using one-way analysis of variance (ANOVA) where a value of $p < 0.05$ was considered statistically significant. This was followed by a pairwise Tukey–Kramer post hoc comparison to determine which IMS beads have recoveries that are significantly different.

RESULTS

Light scatter properties of the beads

Flow cytometry analyses were conducted to characterise the magnetic beads included in the IMS kits. The density dot plots in Figure 1 show the light scatter characteristics of each type of IMS bead.

Flow cytometry analyses of the IMS beads based on the FSC and SSC parameters are indicative of the particle size and refractive properties (Figure 1). BioPoint and TCS beads have higher FSC and SSC values compared to the other two IMS beads, which indicates a larger bead size. The lowest FSC and SSC values are observed in the Waterborne beads, showing that these beads are the smallest in size. Dynabeads exhibit the tightest population among all samples, denoting that these particles are highly uniform in size (Šafařík & Šafaříková 1999). The TCS Isolate beads and the Waterborne beads showed a significant number of aggregates representing 51% (TCS) and 77% (Waterborne) of the total particle numbers detected.

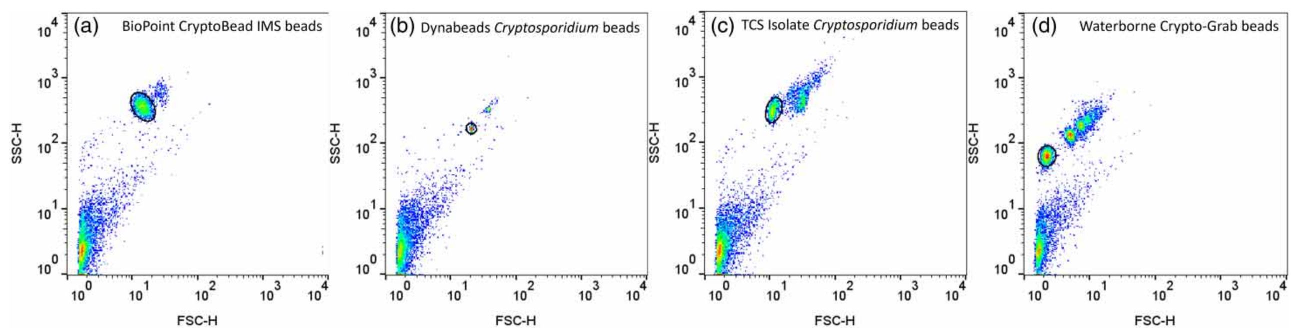


Figure 1 | Flow cytometry density dot plots showing FSC and SSC of the IMS beads. The density dot plots show the main populations of the (a) BioPoint beads, (b) Dynabeads, (c) TCS beads, and (d) Waterborne beads. The FSC parameter is shown on the *x*-axis, while the SSC is on the *y*-axis.

Downloaded from http://iwaponline.com/jwh/article-pdf/21/10/1580/1312367/jwh0211580.pdf
by guest

Analysis of antibodies coated on the beads

The reactivity of the beads with two FITC-labelled secondary antibodies is presented in Figure 2. The immunoglobulin IgG whole molecule secondary antibody reacts with all subclasses of mouse monoclonals, and the immunoglobulin IgM-specific antibody reacts with the μ-chain that is only present on IgM antibodies.

There was a large degree of variation between the different bead types in reactivity with the IgG whole molecule antibody. The BioPoint beads appeared densely coated with IgG antibody. These beads are, in fact, coated with an IgG1 antibody (Weir *et al.* 2000). The Dynabeads appear to be coated with an IgM antibody at a low level, while the TCS beads appear to be coated with a medium level of IgG antibody. The Waterborne beads appear to be coated with a very low level of IgM antibody.

Bead concentrations

The concentrations of the magnetic particles in suspension have not been provided by the manufacturers. Flow cytometry analyses were hence conducted to estimate the number of beads per mL of solution for each bead type with the results presented in Figure 3. Dynabeads, BioPoint beads, and TCS beads all contain relatively similar bead concentrations, whereas Waterborne beads are at a concentration that is nearly 10 times higher than the other beads.

Capture efficiencies at different time points

The ability of the beads to capture 1,000 oocysts in a 1 mL reagent water sample was tested after various incubation periods. Figure 4 shows the percentage of spiked oocysts detected in the supernatant after 5, 10, and 30 min of incubation time.

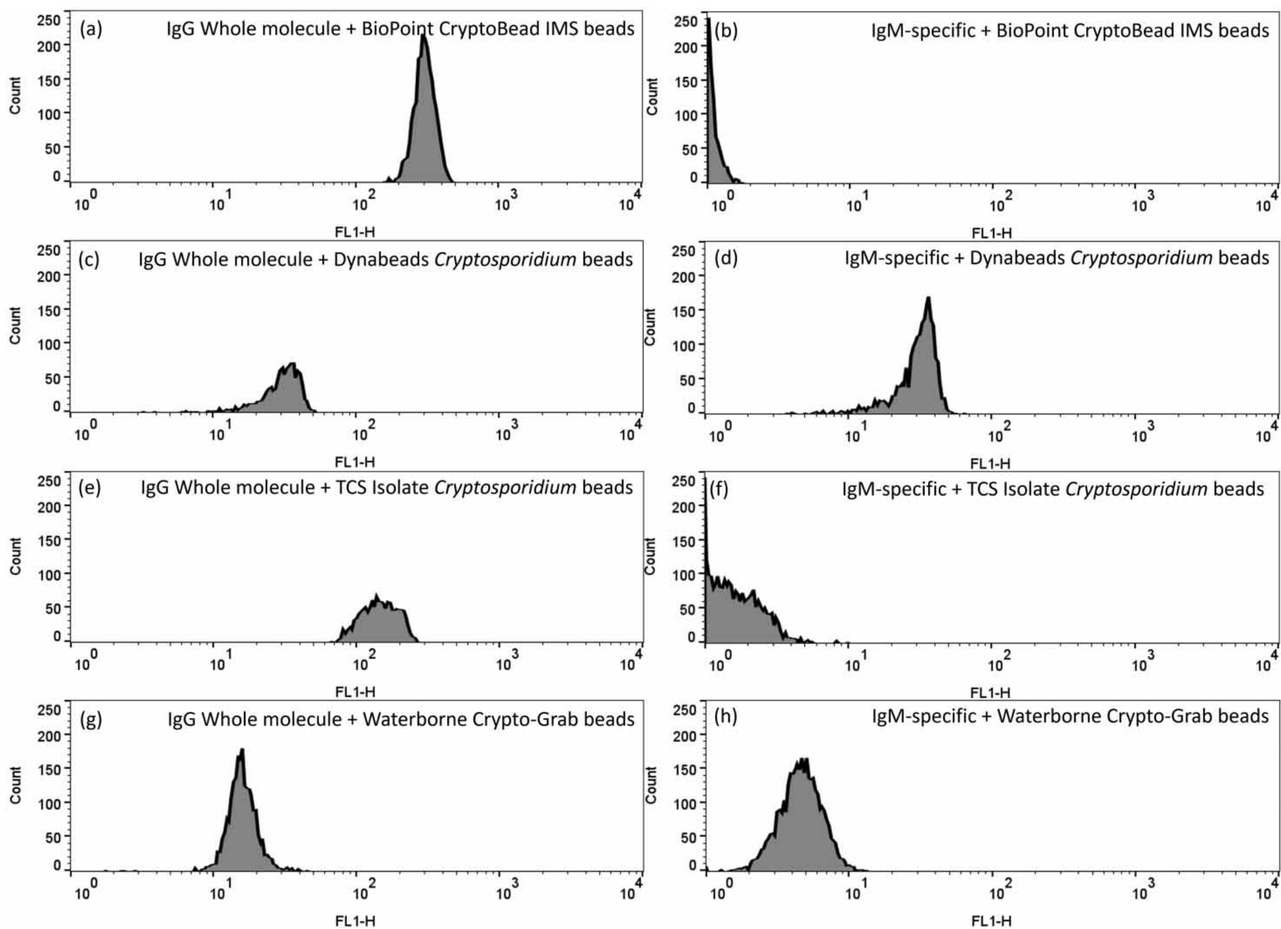


Figure 2 | FL-1 histograms of IMS beads against an anti-mouse FITC IgG whole molecule and IgM-specific antibodies. The histograms are showing the fluorescence intensities of the BioPoint beads when tested against anti-mouse FITC IgG whole molecule antibody (a) and anti-mouse FITC IgM-specific antibody (b). Histograms (c) and (d) display the reaction of Dynabeads, (e) and (f) for TCS beads, and (g) and (h) for Waterborne beads, respectively.

Downloaded from http://iwaponline.com/jwh/article-pdf/21/10/1580/1312367/jwh0211580.pdf
by guest

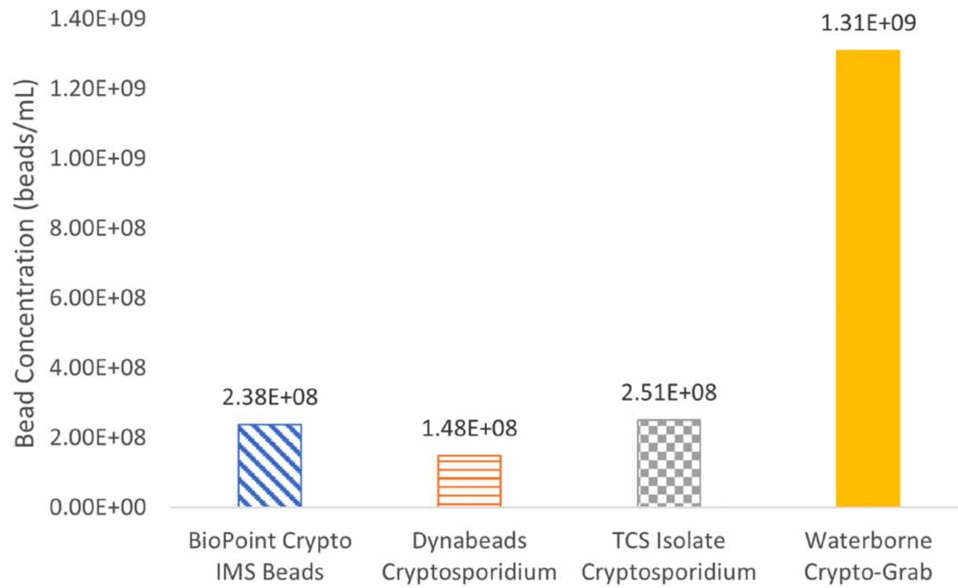


Figure 3 | Calculated concentration of IMS beads from flow cytometry analyses. The bead concentration (given in beads/mL) of the BioPoint beads (in blue diagonal stripes), Dynabeads (in orange horizontal stripes), TCS beads (in grey checkered), and Waterborne beads (in yellow block) are shown after flow cytometry counts.

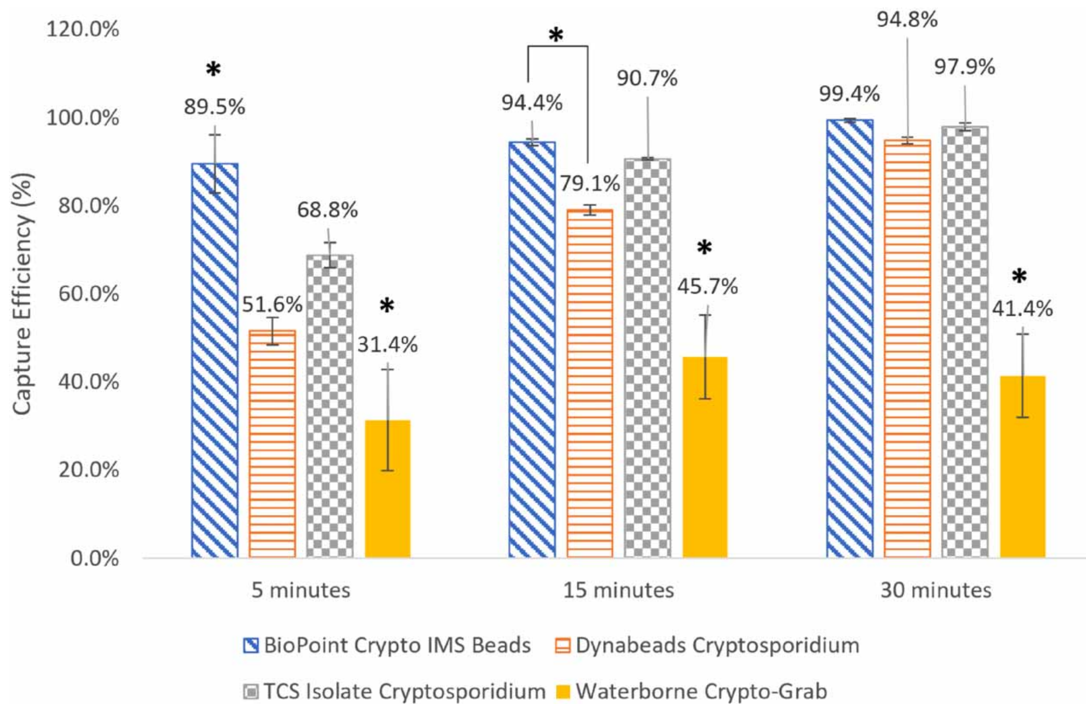


Figure 4 | Capture efficiencies of the IMS beads after incubation times of 5, 15, and 30 min. The capture efficiencies of the BioPoint beads (in blue diagonal stripes), Dynabeads (in orange horizontal stripes), TCS beads (in grey checkered), and Waterborne beads (in yellow block) are shown after incubation times of 5, 15, and 30 min. The average recoveries are presented in percentages with the standard deviation indicated in error bars. * indicate statistically significant results ($p < 0.05$).

Downloaded from http://iwaponline.com/jwh/article-pdf/21/10/1580/1312367/jwh0211580.pdf
by guest

The BioPoint beads showed faster capture efficiency over the shorter time periods than the other beads. The BioPoint, Dynabeads, and TCS beads all achieved a high capture efficiency at the 30-min time point at >94%, while Waterborne beads at 41.4% failed to capture most of the oocysts.

Recovery from different sample types

The recovery efficiencies of the IMS beads were tested against three sample matrices: Cockle Creek sediment is a river water sediment; Fuller's Earth Clay is an artificial clay sample that has been used for proficiency testing; and QC mud is a filter backwash sample from a water treatment plant. The results obtained are presented in Figure 5.

The Dynabeads, TCS, and BioPoint beads all performed well against all sample types. Two sets of analyses with reagent-grade water were carried out to compare the differences between staining with Aqua-Glo™ and EasyStain™ where a similar trend in the beads' results was observed. The Dynabeads showed a lower recovery with the Fuller's Earth Clay sample. The BioPoint beads performed the best across all four sample matrices with the recovery efficiencies ranging from 75 to 93%. In contrast, Waterborne beads had the lowest recovery efficiencies ranging from 0 to 8%.

Purity from different sample matrices

The four different sample matrices resulted in a varying amount of background debris that is present during the microscopy examination of the stained samples. Figure 6 shows a typical field of view from the fluorescent microscopy for each sample matrix analysed with the BioPoint beads, Dynabeads, TCS beads, and Waterborne beads. The high background and the presence of debris and particulate matter such as those observed in Cockle Creek Sediment and Fuller's Earth Clay matrix affect the IMS recoveries as these obscure the oocysts, hence leading to lower yield.

DISCUSSION

The efficiency of the IMS process is influenced by several factors, including the rate and specificity at which the beads bind to the oocysts, the number of contaminating particles present in the sample; the number of magnetic beads used; and the incubation time allowed for the capture of the target organism (Wang *et al.* 2020). Additional operation parameters that affect the recovery efficiency are the magnetic capture strength and the dissociation step, wherein magnetic beads are separated from the target oocysts with heat or acid treatment (Robertson & Gjerde 2001; Andreoli & Sabogal-Paz 2021).

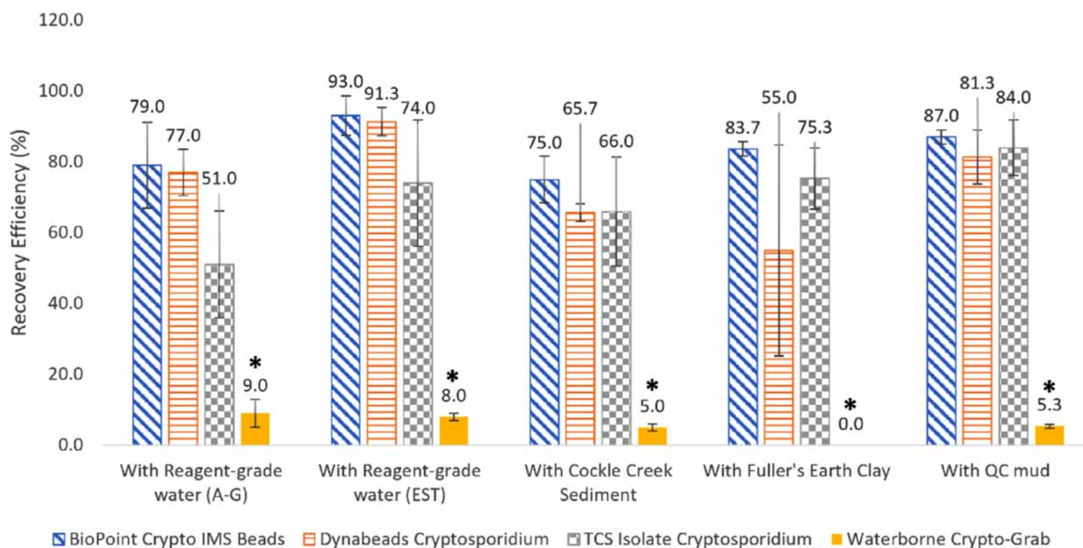


Figure 5 | Recovery efficiencies of the IMS beads against different sample matrices. The percent recoveries of the BioPoint beads (in blue diagonal stripes), Dynabeads (in orange horizontal stripes), TCS beads (in grey checkered), and Waterborne beads (in yellow block) are shown after IMS processing with reagent-grade water and stained with either Aqua-Glo™ or EasyStain™, Cockle Creek Sediment, Fuller's Earth Clay, and QC mud. The average recoveries are presented in percentages with the standard deviation as indicated in error bars. * indicate statistically significant results ($p < 0.05$).

Downloaded from http://iwaponline.com/jwh/article-pdf/21/10/1580/1312367/jwh0211580.pdf
by guest

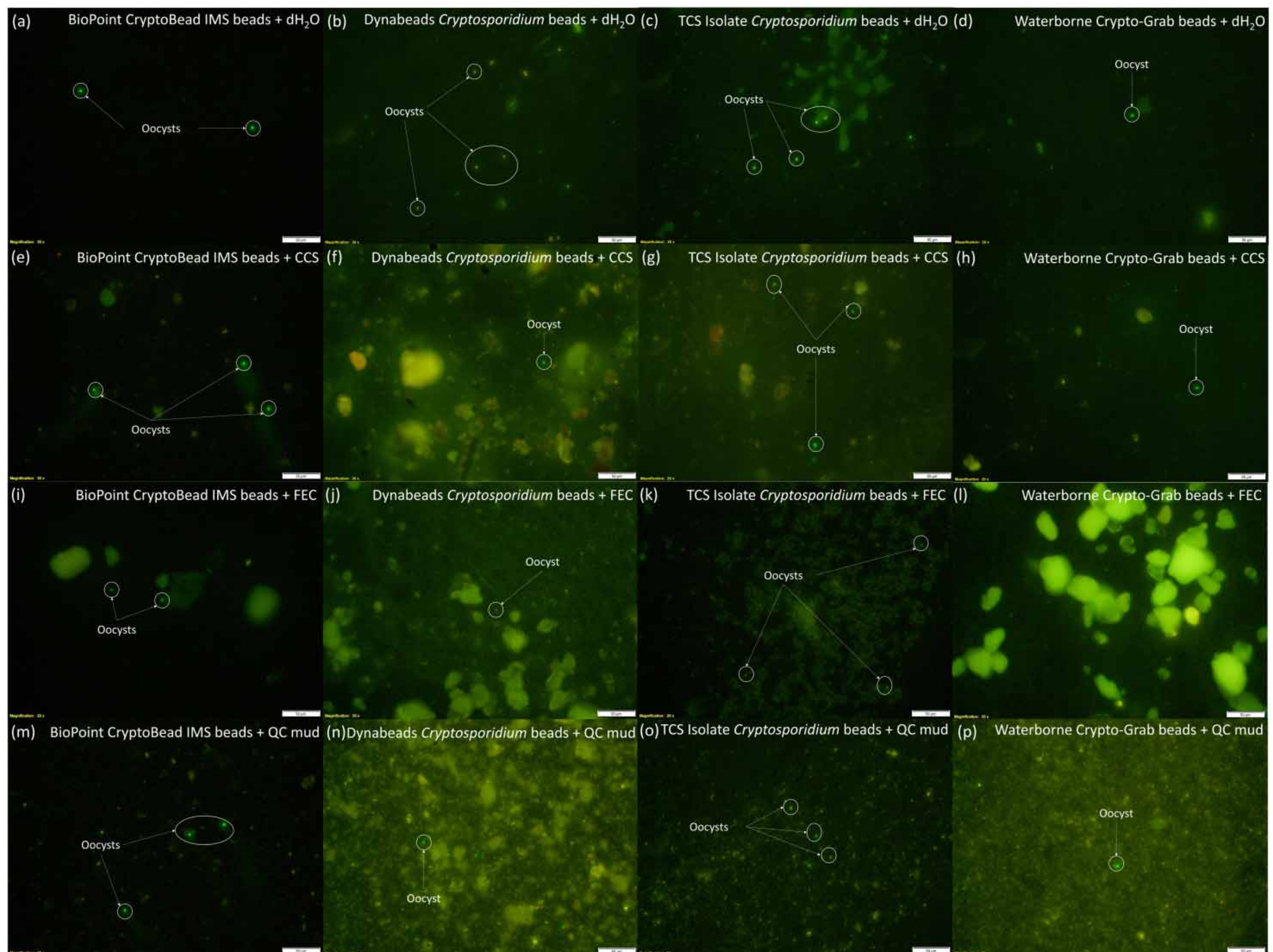


Figure 6 | Post IMS membranes analysed by fluorescence microscopy. The figure shows the membranes following IMS processing with different beads and immunofluorescence staining. Analysed at 20× magnification under the FITC filter, images show the IMS dissociation supernatant for BioPoint beads, Dynabeads, TCS beads, and Waterborne beads after processing reagent-grade water (a)–(d), Cockle Creek Sediment (e)–(h), Fuller's Earth Clay (i)–(l), and QC Mud (m)–(p), respectively, and staining with EasyStain™, where debris and other particulates are visibly observed in the latter three sample matrices.

The tendency of an antibody to form a complex with an antigen pertains to its avidity, which is defined as the equilibrium association constant by convention (Greenspan 2023). Reciprocal to this measure of avidity is the equilibrium dissociation constant, determined by the ratio of the antibody dissociation rate or off-rate to the association rate or on-rate, which measures the velocity of how quickly the antibody dissociates or binds to the target, respectively (Greenspan 2023). The specificity, avidity, and the on-rate of the antibody used will influence the performance of the IMS beads as well as the amount of antibody coated onto the beads. The most widely available *Cryptosporidium*-specific monoclonal antibodies are IgM or IgG3 antibodies (Smith & Nichols 2010). Both IgM and IgG3 monoclonal antibodies are often 'sticky' and have slower on-rates and lower avidity than IgG1 monoclonal antibodies, causing the non-specific binding of this antibody to algal and mineral particles when testing water samples (Vesey *et al.* 1997; Weir *et al.* 2000).

Flow cytometry analysis of the different beads after staining with secondary antibodies shows that Dynabeads and Waterborne beads are coated with IgM antibodies, whereas BioPoint beads and TCS Isolate *Cryptosporidium* both have IgG antibodies. These results are congruent with previously published studies where it was identified that the Dynabeads have an IgM antibody and the TCS Isolate beads have an IgG3 antibody (Smith & Nichols 2010). BioPoint beads have an IgG1 antibody coated onto the magnetic particles, producing beads that have a higher affinity to the surface antigens of *Cryptosporidium* oocysts in water samples. The BioPoint beads showed a high capture efficiency even with a short incubation

Downloaded from http://iwaponline.com/jwh/article-pdf/21/10/1580/1312367/jwh0211580.pdf
by guest

time of 5 min. This is probably due to the combined effect of the superior IgG1 antibody and the high concentration of antibody on the beads.

As determined through flow cytometry analyses, Dynabeads, TCS, and BioPoint beads were relatively similar in size and were supplied at similar bead concentrations. The Waterborne beads were found to be much smaller and were supplied at almost a 10-fold higher concentration. The amount of antibodies on the beads varied significantly. There was 10-fold more antibody on the BioPoint beads than the Dynabeads and double the amount of the TCS beads. The efficiency of immunocapture and magnetic separation is also affected by the particle size, where the large surface area of smaller beads should provide more antibody binding sites and allow more particles to interact with target organisms (Wang *et al.* 2020). This was, however, not observed with the Waterborne beads, where despite the high particle concentration there were very low levels of antibody detected on the beads, possibly causing the poor performance of these beads.

The four IMS kits utilised in this comparative study each have their specific manufacturer's instructions for use. Simply put, the whole IMS process can be divided into four sections: (1) mixing the sample with buffers and beads, (2) incubation to allow magnetic beads to bind to oocysts, (3) washing the sample from the tube to a microcentrifuge tube, and (4) dissociation of the oocysts from the beads, prior to staining and microscopic analyses. The main difference in the methods among the IMS kits was during the incubation and capture of beads. Dynabeads, TCS, and Waterborne beads all recommend a minimum of 1 h incubation with rotation at 15–20 rpm at room temperature, while BioPoint beads require a minimum of 15 min. The BioPoint protocol recommends performing the IMS capture in a 15 mL plastic centrifuge tube, whereas the other beads kits all recommend using a glass Leighton tube. Additionally, both Dynabeads and Waterborne protocols recommend the use of the Dynal® MPC®-1/MPC®-6 and Dynal® MPC®-S magnets (Dynal) for the separation and doing the captures manually by rocking the tube by hand end-to-end at ~90° for 2 min with ~1 tilt/s. BioPoint beads and TCS beads protocols, on the other hand, use an automated capture where magnetic tube holders are rotated at 20 rpm for 5 min and then the supernatant is immediately tipped off. All four kits have the same dissociation process wherein 50 μL of 0.1 M HCl was added to the bead–oocyst pellet and magnetic separation was carried out following vortex mixing and sample incubation. This procedure during the IMS process is a crucial step as having oocysts remaining adherent to the beads following dissociation has become the main cause of reduced oocyst recovery (de Oliveira Pinto *et al.* 2016). It is ideal that a complete dissociation of the bead–oocyst complex occurs to obtain optimal recovery efficiency.

Resulting recovery efficiencies are highly influenced by both the methodology for IMS and the particularities inherent to the sample matrix of interest. There is then a need to evaluate IMS in different matrices such as those from natural sources like rivers and lakes as the efficiency of the U.S. Environmental Protection Agency (EPA) Method 1623.1 has been optimised for drinking water samples but not for complex matrices (Giglio & Sabogal-Paz 2018; Andreoli & Sabogal-Paz 2021). The selection of river sediment, clay samples, and filter backwash samples as the matrices of interest for this study is based on the complexity of these samples and the likelihood of such being analysed in water testing laboratories. Out of the three matrices, the Cockle Creek Sediment appeared to contain some magnetic debris. It is possible that this matrix had dissolved iron, the most common type of interference from both surface water and groundwater, in the form of either ferrous or ferric aqueous ionic species (Yakub & Stadterman-Knauer 2000). In solution, insoluble iron oxides and hydroxides are formed and further influence the overall turbidity of the matrix. (Yakub & Stadterman-Knauer 2000).

Interference in the separation of oocysts can be influenced by the turbidity of both organic and inorganic debris present in the sample such as clays and coagulants arising from the source waters or from additional treatment processes prior to IMS (U.S. EPA 2012). The Dynabeads showed variable recoveries with the Fuller's Earth Clay matrix. Previous studies have reported problems using Dynabeads with clay samples (McElroy *et al.* 2001).

Additionally, it must be noted that there is still a research gap in detecting oocyst present in filter backwash samples from high turbidity water samples (Ogura & Sabogal-Paz 2022). In water treatment plants, although most *Cryptosporidium* oocysts are removed during the settling and filtration stages, the small size and compressibility of the oocysts enable some to pass through (Ogura & Sabogal-Paz 2022). Similarly, recirculating the filter backwash water reintroduces the oocysts in the treatment process. Relatively good recoveries at ~80% from BioPoint, Dynabeads, and TCS Isolate beads were still achieved despite the presence of particles that may adhere to oocysts, as commonly seen in filter backwash water samples (Ogura & Sabogal-Paz 2022).

After IMS processing, the oocysts dissociated from immunomagnetic beads are visualised under fluorescence microscopy. Manual counting of cells under fluorescence microscopy requires trained personnel and can be time-consuming, labour-intensive, and prone to errors (Aldughayfiq *et al.* 2023). The use of immunofluorescent staining enables the marked distinction of *Cryptosporidium* oocysts from the non-fluorescent background and makes the microscopic investigation easier (O'Leary *et al.*

Downloaded from http://iwaponline.com/jwh/article-pdf/21/10/1580/1312367/jwh0211580.pdf
by guest

2021). However, debris from IMS processing is often auto-fluorescent and can obscure the target cells, as shown in the Fuller's Earth Clay samples. Out of the four IMS kits tested, the resulting slides from the BioPoint beads had the least background and debris. The quality of the processed sample is most likely influenced by the type of antibody present on the IMS beads, in which the identified IgG1-coated beads are less prone to non-specific binding, compared to IgM and IgG3 antibodies. If a large amount of debris is present, then it is difficult to visualise oocysts and can reduce recovery efficiencies.

The presence of various materials such as sediments, dissolved metals, and particulates in sample matrices can hinder achieving optimal recovery efficiency in IMS. Conducting IMS against the different sample matrices tested the robustness of the different IMS bead kits and exhibited how both the method and the sample matrix affect the resulting slides and oocyst recoveries post-IMS processing.

CONCLUSIONS

Two of the IMS bead kits, Dynabeads™ *Cryptosporidium* and Waterborne Crypto-Grab™, have both been found to be coated with IgM antibodies. BioPoint beads and TCS Isolate beads are both coated with IgG antibodies, resulting in beads with higher affinity to oocyst samples. Among the four IMS bead kits, the BioPoint beads were the best performing when tested across different matrices both in terms of recovery efficiency and the quality of membranes following IMS. Fluorescence microscopy visualisation and enumeration of the recovered oocysts are influenced by both the method and the sample matrix processed. As there is a lack of documented protocols on how complex matrices should be analysed, it is highly recommended that further research and extensive testing be carried out to standardise and tailor these methods for various environmental water samples.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

J.C.A. was employed by BioPoint Pty. Ltd, which has provided their products for this study. The outcomes of this research may lead to the development of products that may be licenced to the same company.

REFERENCES

Aldughayfiq, B., Ashfaq, F., Jhanjhi, N. Z. & Humayun, M. 2023 YOLOv5-FPN: a robust framework for multi-sized cell counting in fluorescence images. *Diagnostics* **13** (13), 2280.

Andreoli, F. C. & Sabogal-Paz, L. P. 2019 Coagulation, flocculation, dissolved air flotation and filtration in the removal of *Giardia* spp. and *Cryptosporidium* spp. from water supply. *Environmental Technology* **40** (5), 654–663.

Andreoli, F. C. & Sabogal-Paz, L. P. 2021 Detection of *Giardia* and *Cryptosporidium* in environmental matrices with immunomagnetic separation: two or three acid dissociations. *Parasitology Research* **120** (2), 629–635.

Costa, D., Razakandrainibe, R., Basmaciyan, L., Raibaut, J., Delaunay, P., Morio, F., Gargala, G., Villier, V., Mouhajir, A., Levy, B. & Rieder, C. 2022 A summary of cryptosporidiosis outbreaks reported in France and overseas departments, 2017–2020. *Food and Waterborne Parasitology* **27**, e00160.

de Oliveira Pinto, D., Urbano, L. & Neto, R. C. 2016 Immunomagnetic separation study applied to detection of *Giardia* spp. cysts and *Cryptosporidium* spp. oocysts in water samples. *Water Science and Technology: Water Supply* **16** (1), 144–149.

Giglio, G. L. & Sabogal-Paz, L. P. 2018 Performance comparison of three methods for detection of *Giardia* spp. cysts and *Cryptosporidium* spp. oocysts in drinking-water treatment sludge. *Environmental Monitoring and Assessment* **190**, 1–10.

Greenspan, N. S. 2023 Epitopes, paratopes, and other topes 30 years on: understanding what we are talking about. *Human Immunology* **84** (9), 429–438.

Hassan, E. M., Örmeci, B., DeRosa, M. C., Dixon, B. R., Sattar, S. A. & Iqbal, A. 2021 A review of *Cryptosporidium* spp. and their detection in water. *Water Science and Technology* **83** (1), 1–25.

McElroy, W., Cabello, E. & Pillai, S. D. 2001 Efficacy of an immuno-magnetic separation system for recovering *Cryptosporidium* oocysts from soils. *Journal of Rapid Methods & Automation in Microbiology* **9**, 63–70.

Nime, F. A., Burek, J. D., Page, D. L., Holscher, M. A. & Yardley, J. H. 1976 Acute enterocolitis in a human being infected with the protozoan *Cryptosporidium*. *Gastroenterology* **70** (4), 592–598.

O'Leary, J. K., Sleator, R. D. & Lucey, B. 2021 *Cryptosporidium* spp. diagnosis and research in the 21st century. *Food and Waterborne Parasitology* **24**, e00131.

Ogura, A. P. & Sabogal-Paz, L. P. 2022 Detection of *Cryptosporidium parvum* oocysts in artificially contaminated filter backwash water and ozone treatment at pilot scale. *Ozone: Science & Engineering* **44** (5), 426–437.

Downloaded from http://iwaponline.com/jwh/article-pdf/21/10/1580/1312367/jwh0211580.pdf
by guest

Robertson, L. J. & Gjerde, B. 2001 Factors affecting recovery efficiency in isolation of *Cryptosporidium* oocysts and *Giardia* cysts from vegetables for standard method development. *Journal of Food Protection* **64** (11), 1799–1805.

Ryan, U., Zahedi, A., Feng, Y. & Xiao, L. 2021 An update on zoonotic *Cryptosporidium* species and genotypes in humans. *Animals* **11** (11), 3307.

Šafařík, I. & Šafaříková, M. 1999 Use of magnetic techniques for the isolation of cells. *Journal of Chromatography B: Biomedical Sciences and Applications* **722** (1–2), 33–53.

Smith, H. V. & Nichols, R. A. 2010 *Cryptosporidium*: detection in water and food. *Experimental Parasitology* **124** (1), 61–79.

Temesgen, T. T., Tysnes, K. R. & Robertson, L. J. 2021 Use of oxidative stress responses to determine the efficacy of inactivation treatments on *Cryptosporidium* oocysts. *Microorganisms* **9** (7), 1463.

U.S. EPA. Method 1623.1:2012. 2012 *Cryptosporidium and Giardia in Water by Filtration/IMS/FA*. U.S. Environmental Protection Agency, Office of Water, Washington, DC.

Vesey, G., Deere, D., Weir, C. J., Ashbolt, N., Williams, K. L. & Veal, D. A. 1997 A simple method for evaluating *Cryptosporidium*-specific antibodies used in monitoring environmental water samples. *Letters in Applied Microbiology* **25** (5), 316–320.

Wang, Z., Cai, R., Gao, Z., Yuan, Y. & Yue, T. 2020 Immunomagnetic separation: an effective pretreatment technology for isolation and enrichment in food microorganisms detection. *Comprehensive Reviews in Food Science and Food Safety* **19** (6), 3802–3824.

Warnecke, M., Weir, C. & Vesey, G. 2003 Evaluation of an internal positive control for *Cryptosporidium* and *Giardia* testing in water samples. *Letters in Applied Microbiology* **37** (3), 244–248.

Weir, C., Vesey, G., Slade, M., Ferrari, B., Veal, D. A. & Williams, K. 2000 An immunoglobulin G1 monoclonal antibody highly specific to the wall of *Cryptosporidium* oocysts. *Clinical Diagnostic Laboratory Immunology* **7** (5), 745–750.

Yakub, G. P. & Stadterman-Knauer, K. L. 2000 Evaluation of immunomagnetic separation for recovery of *Cryptosporidium parvum* and *Giardia duodenalis* from high-iron matrices. *Applied and Environmental Microbiology* **66** (8), 3628–3631.

First received 18 July 2023; accepted in revised form 15 September 2023. Available online 28 September 2023

Downloaded from http://iwaponline.com/jwh/article-pdf/21/10/1580/1312367/jwh0211580.pdf
by guest