Comparison of methods for the concentration of *Cryptosporidium* oocysts and *Giardia* cysts from raw waters

Christobel Ferguson, Christine Kaucner, Martin Krogh, Daniel Deere, and Malcolm Warnecke

Abstract: This study compared the recovery of *Cryptosporidium* oocysts and *Giardia* cysts ((oo)cysts) from raw waters using 4 different concentration–elution methods: flatbed membranes, FiltaMax™ foam, Envirochek™ HV capsules, and Hemoflow ultrafilters. The recovery efficiency of the combined immunomagnetic separation and staining steps was also determined. Analysis of variance of arcsine-transformed data demonstrated that recovery of *Cryptosporidium* oocysts by 2 of the methods was statistically equivalent (flatbed filtration 26.7% and Hemoflow 28.3%), with FiltaMax™ and Envirochek™ HV recoveries significantly lower (18.9% and 18.4%). Recovery of *Giardia* cysts was significantly higher using flatbed membrane filtration (42.2%) compared with the other 3 methods (Envirochek™ HV 29.3%, FiltaMax™ 29.0%, and Hemoflow 20.9%). All methods were generally acceptable and are suitable for laboratory use; 2 of the methods are also suitable for field use (FiltaMax™ and Envirochek™ HV). In conclusion, with recoveries generally being statistically equivalent or similar, practical considerations become important in determining which filters to use for particular circumstances. The results indicate that while low-turbidity or "finished" waters can be processed with consistently high recovery efficiencies, recoveries from raw water samples differ significantly with variations in raw water quality. The use of an internal control with each raw water sample is therefore highly recommended.

Key words: catchments, Envirochek™ HV, Hemoflow, FiltaMax™, flatbed filtration.

Résumé : Cette étude a comparé la récupération d'oocystes de *Cryptosporidium* et de sporocystes de *Giardia* à partir d'eaux brutes à l'aide de quatre différentes méthodes d'élution–concentration: membranes horizontales, mousse FiltaMax™, capsules Envirochek™ HV et ultrafiltres Hemoflow. L'efficacité de récupération de la séparation immunomagnétique (SIM) combinée à la coloration a également été déterminée. Une analyse de la variance des données converties en arcsinus a démontré que la récupération d'oocystes de *Cryptosporidium* par deux des méthodes était statistiquement équivalente (filtration horizontale 26,7 %; Hemoflow 28,3 %), les récupérations avec FiltaMax™ et Envirochek™ HV s'avérant significativement inférieures (18,9 et 18,4 %). La récupération de sporocystes de *Giardia* était significativement plus élevée avec la filtration sur membrane horizontale (42,2 %) comparativement aux trois autres méthodes (Envirochek™ HV 29,3 %, FiltaMax™ 29,0 % et Hemoflow 20,9 %). Toutes les méthodes étaient généralement acceptables et conviennent à l'utilisation en laboratoire, deux des méthodes conviennent également à l'utilisation sur le terrain (FiltaMax™ et Envirochek™ HV). En conclusion, lorsque les récupérations sont généralement statistiquement équivalentes ou semblables, les aspects pratiques deviennent importants pour la détermination du filtre à utiliser dans des circonstances particulières. Les résultats indiquent que bien que des eaux à basse turbidité (ou « finies ») peuvent être traitées avec des taux d'efficacité de récupération reproductibles, les récupérations à partir d'échantillons d'eaux brutes diffèrent significativement selon les variations dans la qualité de l'eau brute. L'utilisation d'un témoin interne pour chaque échantillon d'eau brute est par conséquent fortement recommandée.

Mots clés : captage, Envirochek™ HV, Hemoflow, FiltaMax™, filtration horizontale.

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Introduction

Numerous studies have reported the presence of the parasitic protozoans *Cryptosporidium* and *Giardia* in raw and treated drinking waters (Hutton et al. 1995; Karanis et al. 1998; LeChevallier et al. 1991; Rose et al. 1991; Svoboda et al. 1999). Detection of these organisms is usually performed by multiple-stage methods, such as US Environmental Protection Agency (USEPA) method 1623 (USEPA 1999). These typically involve sample concentration by filtration, immunomagnetic separation (IMS), immunofluorescent staining, and then detection and enumeration by epifluorescence microscopy. Use of such methods can result in substantial losses of target organisms during processing, particularly with some raw waters. However, few studies report results adjusted for the recovery efficiency of the analysis. Among the studies that do adjust for recovery, the efficiency is usually calculated from the average recovery of separately spiked samples (LeChevallier et al. 2000; Medema and Schijven 2001; Simmons et al. 2001). The recent commercial availability of an internal control for *Cryptosporidium* and *Giardia* provides an opportunity to quantify recovery efficiency, thus accounting for the effects of the specific matrix and handling conditions of every sample.

A number of products are available for the concentration and elution of *Cryptosporidium* oocysts and *Giardia* cysts from raw and finished waters. Given the different characteristics of these products, they can be expected to be more or less effective for different applications and different sample water types. Flatbed membrane (293 mm in diameter) filtration (Millipore Corp., Bedford, Mass.) is a laboratory-based concentration method with routine local usage since 1993 (Cox et al. 2003). FiltaMax™ foam filter capsules (IDEXX, Laboratories Inc., Westbrook, Maine) use multiple layers of compressed open-cell foam discs to trap target organisms, allow field concentration of samples and subsequent laboratory elution, and are USEPA method 1623 and UK Drinking Water Inspectorate (DWI) approved. Envirochek™ HV sampling capsules (PALL Life Sciences, East Hills, New York) use a pleated 1-µm polyethersulfone membrane, allow field concentration of samples, and are also approved by USEPA method 1623 and UK DWI. The use of Hemoflow ultrafiltration units (Fresenius Medical Care, Bad, Homburg, Germany) for the concentration of *Cryptosporidium* has been described by Simmons et al. (2001) and can potentially be used to simultaneously concentrate samples for a variety of organisms.

Analyses of raw waters for *Giardia* and *Cryptosporidium* frequently give nondetection results or detections at low concentrations. This necessitates the use of methods with quantifiable sensitivity of detection to allow water utility managers to confidently make decisions based on the data. The aims of this study were to (*i*) quantify the recovery efficiencies of *Cryptosporidium* oocysts and *Giardia* cysts from raw waters using flatbed membrane filtration, FiltaMax™ foam filters, Envirochek™ HV sampling capsules, and the Hemoflow ultrafiltration unit utilizing an internal positive control and (*ii*) determine the recovery efficiency of the combined IMS and staining steps and determine if this varied with the use of different concentration methods and (or) between samples.

Methods

Water samples

Raw water samples of varying turbidity, 1–15 nephelometric turbidity units (NTU), were collected from 3 different sites near Sydney, Australia, over a 4-week period. In the first week, a 9000-L volume of low-turbidity surface water (1.1 NTU) was collected from a drinking water reservoir designated site A. The water was transported by tanker to the laboratory where it was pumped into 2 tanks each of 5000-L capacity and stored at ambient temperature. Water was pumped as required from these tanks into a 1000-L container. In week 2, raw water was collected from site B, an urban creek impacted by stormwater (approx. 8 NTU). In week 4, raw water was collected from site C, an urban creek downstream of a sewage treatment plant (approx. 14 NTU). For sites B and C, approx. 300 L of water was collected aseptically into 50-L containers and then mixed in a 500-L container upon arrival at the laboratory. Individual test samples were prepared by pumping approx. 10 L of raw water from the 500-L container into clean disposable polyethylene sample bags (Entapack, Australia). Each sample bag was weighed and a portion of water withdrawn for the analysis of physical and chemical parameters (excluding a small number of samples from site B).

ColorSeed™ (oo)cysts (BTF Pty Ltd., North Ryde, Australia) were utilized as an internal control, and a vial was added to each raw water sample 18 h prior to analysis to allow estimation of the percent recovery of each method in its entirety. ColorSeed™ vials contained 98 ± 1.0 *Cryptosporidium* oocysts and 98 ± 1.2 *Giardia* cysts that had been permanently colour modified prior to flow cytometry sorting such that they fluoresced red when viewed using a green filter block on an epifluorescence microscope. This colour modification of the ColorSeed™ (oo)cysts enables them to be readily distinguished from naturally occurring (oo)cysts and unmodified spiked (oo)cysts when using fluorescence microscopy. Each ColorSeed™ vial was vortexed for 30 s and the contents decanted into the sample. The vial was then rinsed twice with 3 mL of elution buffer No. 1 (0.05% Tween 80 (ICN Biomedicals Inc., Costa Mesa, Calif.) and 2 mmol/L tetrasodium pyrophosphate (Sigma–Aldrich Corp., St. Louis, Mo.), adjusted to pH 8), vortexed for 30 s, and decanted into the sample.

Control samples of approx. 10 L were prepared from deionized water. ColorSeed™ was added to some samples as an internal control in the same manner as described for the raw water samples. Some samples remained uninoculated as a check for possible cross-contamination between samples (see Fig. 1 for an outline of the experimental design).

Concentration–elution procedures

Four different filtration methods were used for the concentration of *Cryptosporidium* oocysts and *Giardia* cysts from a variety of water matrices. The experimental design is shown in Fig. 1. Six approx. 10-L samples of raw water and 4 approx. 10-L samples of deionized water were concen-

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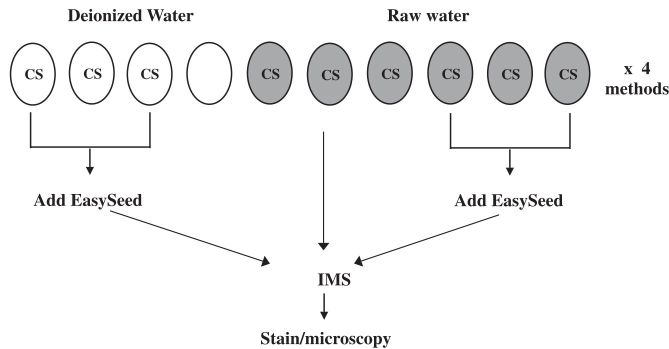
Fig. 1. Experimental design for the preparation of forty 10-L water samples each week. Each week, 4 sets of samples 1–10 were prepared and 1 set analysed by each of the filtration methods (flatbed membrane filtration, FiltaMax^{TM} foam, Envirochek^{TM} HV capsules, and Hemoflow ultrafiltration cartridges). All 4 sets were processed within a single working day. A different raw water was tested each week (CS, ColorSeed^{TM}).



trated by each of the 4 methods within the same working day. The deionized water controls were included to evaluate the reproducibility of each method over the 4-week period (spiked controls) and to test for the possibility of cross-contamination between samples (unspiked controls). The methodologies for the filtration methods are described as follows.

Flatbed membrane filtration

This method was derived from that originally described by Ongerth and Stibbs (1987). Water samples were concentrated through a 293-mm-diameter flatbed filter unit (Millipore) containing a 2-µm pore size track-etched polycarbonate membrane (Osmonics, Massachusetts) using a peristaltic water pump (Watson Marlow unit 604S) at a rate not exceeding 4 L/min. The membrane was transferred to a modified perspex sheet (Micronics, Sydney, Australia), sprayed with elution buffer No. 1, and the concentrate eluted using a rubber squeegee (Micronics) and collected in a 50-mL centrifuge tube (Iwaki). This elution procedure was repeated 2 to 4 times, following which the membrane was removed and discarded and the perspex sheet and squeegee further rinsed with elution buffer. The filter wash was centrifuged at 1620*g* for 10 min at 4 ± 2 °C. The supernatant was aspirated to leave the pellet remaining in a volume of 5 mL, which was resuspended by vortexing. The flatbed apparatus was cleaned between samples by backflushing with a detergent solution (Tergazyme, Alconox Inc., White Plains, New York) followed by water. The perspex screen and the squeegee were scrubbed with detergent and rinsed with water. Sample processing time for flatbed filtration was approx. 5 h for 10 samples.

FiltaMax^{TM}

Samples were filtered through FiltaMax^{TM} foam filters using a peristaltic water pump (Watson Marlow unit 604S) at a rate not exceeding 4 L/min, as required by the manufacturer's instructions (IDEXX 2000). The foam filters were then removed from the filter housing and eluted by compressing and decompressing the filters 20 times in an elution buffer of 0.1% Tween 20 in phosphate-buffered saline (PBST) using a FiltaMax^{TM} manual wash station (IDEXX). The foam pads were washed a second time with expansion–compression cycles in the elution buffer. After each round of expansion–compression, the sample eluate was concentrated further by filtration of the sample through a 3-µm cellulose nitrate membrane filter (IDEXX) and the remaining volume added to a centrifuge tube. The membrane was washed in a 5-mL volume of PBST and the washings added to the centrifuge tube. On some occasions, multiple membranes were required. The samples were then centrifuged at 1100*g* for 15 min and the supernatant aspirated to approx. 5 mL (DWI 1999). Sample processing time for the FiltaMax^{TM} with a single manual wash station was approx. 9 h for 10 samples.

Envirochek^{TM} HV

The Envirochek^{TM} HV sampling capsule (PALL Life Sciences) is a 1-µm absolute pore size pleated filter in a polycarbonate housing. Samples were filtered through the capsule using a peristaltic water pump (Watson Marlow unit 604S) at a rate no greater than 2 L/min, as required by the manufacturer's instructions (PALL Life Sciences 2000). Oocysts and cysts were eluted from the capsule filter based on the method of Pezzana et al. (2000). This involved the addition of 125 mL of an elution buffer (150 µL of Tween 80 (ICN Biomedicals) and 150 µL of antifoam A (Sigma–Aldrich Corp.) per litre of PBS (Sigma–Aldrich)) to the capsule filter, which was then horizontally agitated on a wrist-action shaker for 5 min with the bleed valve in the 12 o'clock position. The eluate was then decanted into a 250-mL centrifuge tube and a second 125-mL volume of elution buffer added to the capsule filter. This was agitated with a wrist-action shaker for 5 min with the bleed valve in the 4 o'clock position and a further 5 min with the bleed valve in the 8 o'clock position before being decanted into the same 250-mL centrifuge tube and centrifuged at 3000*g* for 30 min at 4 ± 2 °C. Vacuum aspiration was used to remove all but 5 mL of the supernatant and the pellet was resuspended by vortexing. Processing time for 10 samples using a wrist-action shaker capable of shaking 4 capsules simultaneously was approx. 5 h.

Hollow fiber ultrafiltration

The Hemoflow F80S ultrafilter (80 000 molecular mass cut-off) (Fresenius Medical Care) is a polysulfone hollow fiber unit contained within a polycarbonate housing. Ten-litre samples were recirculated through the filter at a pressure of approx. 7 psi (1 psi = 6.895 kPa) using a peristaltic water pump (Cole-Parmer 7591-55 with Masterflex 7019-32 pump head). When the volume of the sample was reduced to the volume within the filter capsule and the tubing, the inlet and outlet hoses were placed into a 250-mL centrifuge tube containing 200 mL of elution buffer (1% Laureth-12 (Pall Life Sciences) in 100 mmol/L PBS and 150 µL of Antifoam A (Sigma–Aldrich) per litre of PBS). The buffer was then recirculated at a pressure of <5 psi until a volume of approx. 75 mL remained in the centrifuge tube. The volume within the capsule and tubing was then collected in the centrifuge tube and any additional liquid purged with air pressure. The concentrate was then centrifuged at 3000*g* for 30 min at 4 ± 2 °C and the supernatant was vacuum aspirated until approx.

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5 mL remained. The pellet was resuspended by vortexing. Sample processing time for 10 samples with 2 samples being concentrated simultaneously was 8 h.

Purification procedures

After concentration, samples were processed using IMS and immunofluorescent antibody staining, with 4′,6-diamidino-2-phenylindole (DAPI) staining for confirmation of (oo)cysts. The entire pellet produced from each sample was processed and examined. Packed pellets ranged in size from <0.1 to 1 mL in volume, with those >0.5 mL being split into 2 IMS tests (as per the manufacturer's instructions).

EasySeed™ (containing 98 ± 1.4 *Cryptosporidium* oocysts and 98 ± 1.4 *Giardia* cysts) was spiked into some samples (as described in Fig. 1) immediately prior to IMS to quantify losses associated with the IMS and staining procedures. EasySeed™ vials were vortexed for 30 s before being added to an IMS tube. One millilitre of the 10× SL-buffer A from the IMS kit was then added to the vial, vortexed for 30 s, and the washings added to the IMS tube. One millilitre of the 10× SL-buffer B from the IMS kit was then added to the vial, vortexed for 30 s, and decanted into the IMS tube. The sample concentrate was then added to the IMS tube.

IMS was performed using the Dynabeads GC-Combo kit (Dynal, Oslo) according to the manufacturer's instructions, except that following the acid dissociation step, the sample was immediately transferred to a 13-mm polycarbonate membrane filter of 0.8-μm pore size (Nuclepore, Clifton, New Jersey) mounted on a vacuum manifold. The organisms on the membrane were treated with methanol for 1 min and then overlaid for 2 min with 80 μL of DAPI (2 μg/mL in PBS) (Sigma–Aldrich). The DAPI was removed by vacuum filtration and the membrane washed with 200 μL of EasyStain™ wash buffer (BTF Pty Ltd) and then overlaid for 15 min with 80 μL of EasyStain™ containing fluorescein isothiocyanate labelled antibodies specific for *Cryptosporidium* and *Giardia* (Weir et al. 2000). All staining was performed at room temperature. The membrane was then transferred to a slide, overlaid with the EasyStain™ mounting medium (BTF Pty Ltd.), and sealed with a coverslip. The slide-mounted membrane was then examined by epifluorescence microscopy.

Microscopy

Sample slides were examined using an epifluorescence microscope (Zeiss Axioskop). The membrane surface was scanned at 200× power for *Cryptosporidium* oocysts and *Giardia* cysts using a blue filter set (450–490 nm excitation, 510-nm dichroic beam-splitting mirror, and 520-nm-long pass filter) for fluorescein isothiocyanate fluorescence. All (oo)cysts were viewed using a green filter block (540–580 nm excitation, 595-nm dichroic beam-splitting mirror, and 610–680 nm suppression filter) to distinguish ColorSeed™ (oo)cysts from unspiked and EasySeed™ organisms. When necessary, objects were further examined at 400× power and with a DAPI (UV) filter set (340–380 nm excitation, 400-nm dichroic beam-splitting mirror, and 420-nm suppression filter) (USEPA 1999) to allow visualization of nuclei to assist with identification.

Statistical analysis

Results for the recovery of ColorSeed™ *Cryptosporidium* and *Giardia* (oo)cysts were analysed using a two-factor analysis of variance (ANOVA) as implemented in the GLM procedure of SAS (SAS Institute Inc., Cary, North Carolina). Factors used were method (Envirochek™ HV, FiltaMax™, flatbed, and Hemoflow) and sample (water sample from sites A, B, and C) and the interaction of these 2 factors. All factors were assumed to be fixed; significance of factors was determined using a type I error rate of 0.05. Based on analysis of the residuals from the ANOVA, there was some evidence of heterogeneous variances and nonnormal distribution of residuals when the raw data were analysed. All data were subsequently arcsine transformed prior to analysis and the analyses redone. This improved the pattern of residuals in terms of homogeneity of variance but there was still some evidence of nonnormal distribution of residuals. Student–Newman–Keuls multiple comparison tests were used to compare means for each method. The tables present the means and standard deviations calculated from the raw data analysis, while Student–Newman–Keuls groupings were derived from the arcsine-transformed data. Since the results for week 1 suggested an unusually low recovery of *Cryptosporidium* oocysts for the Hemoflow method (and this was the first time that this technique had been used in this laboratory), the above analyses were repeated after dropping the first week's results. Dropping these results led to improvements in homogeneity of variance and normality of distribution of residuals.

Results

Tables 1 and 2 show the mean percent recovery of ColorSeed™ *Cryptosporidium* and *Giardia* (oo)cysts from deionized water for the various test weeks and for each of the filtration methods. Deionized water samples were tested each week to compare operator efficiency between weeks. Table 1 shows that 3 of the methods were performed consistently each week. The Hemoflow method showed a change in recovery efficiency over time, with week 1 having significantly lower recovery than the following 2 weeks (Table 1). Although this had been anticipated, as the method was new to our laboratory, it was difficult to avoid, since no experienced operator was available. Other concentration methods were performed by experienced operators and recovery efficiency showed no interaction with time (Table 1). Consequently, the data for raw water samples was analysed with and without week 1 for comparison.

Table 2 shows that the mean recovery of *Cryptosporidium* oocysts from deionized water was significantly higher using flatbed membrane filtration (72.8%) compared with the other 3 methods. Envirochek™ HV showed the next highest recovery efficiency (64.4%), which was also significantly higher than was achieved with the other 2 methods. The highest mean recovery of *Giardia* cysts from deionized water was obtained using flatbed filtration and Hemoflow ultrafiltration cartridges (39.0% and 36.1%, respectively). Both methods gave significantly higher results than the Envirochek™ HV and FiltaMax™ (Table 2).

Analysis of all raw water data indicated that flatbed filtration had significantly higher recovery efficiency for ColorSeed™

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Table 1. Mean percent recovery and SD of ColorSeed™ *Cryptosporidium* oocysts and *Giardia* cysts from deionized water by all methods comparing weeks 1, 2, and 3.

	Method											
	Envirochek™ HV			FiltaMax™			Flatbed filtration			Hemoflow		
Week	Mean	SD	SNK	Mean	SD	SNK	Mean	SD	SNK	Mean	SD	SNK
Cryptosporidium												
1	64.3	7.4	A	42.2	2.1	A	84.0	7.2	A	8.83	9.3	A
2	60.9	5.2	A	47.3	14.3	A	67.4	9.1	A	55.1	10.1	B
3	68.0	8.9	A	36.2	6.5	A	67.0	7.8	A	63.9	16.6	B
Giardia												
1	18.7	4.6	A	16.0	5.1	A	37.1	11.4	A	13.9	4.8	A
2	26.9	6.0	A	26.5	2.0	A	41.5	2.9	A	38.4	7.2	B
3	16.7	7.7	A	21.9	7.0	A	38.4	9.2	A	56.1	13.5	B

Note: SNK, grouping by the Student–Newman–Keuls test (arcsine-transformed data). Different letters indicate a significant difference at the $p < 0.05$ level. $n = 3$ for all samples except FiltaMax week 3 where $n = 2$.

Table 2. Mean percent recovery and SD of ColorSeed™ *Cryptosporidium* oocysts and *Giardia* cysts from deionized water for each filtration method and sample week (two-way ANOVA).

	Cryptosporidium oocysts				*Giardia* cysts			
Test	Mean	SD	*n*	SNK	Mean	SD	*n*	SNK
Method								
Flatbed filtration	72.8	7.1	9	A	39.0	7.8	9	A
Envirochek™ HV	64.4	9.3	9	B	20.8	7.1	9	B
Hemoflow	62.6	10.9	9	C	36.1	20.0	9	A
FiltaMax™	42.6	27.8	8	C	21.4	6.5	8	B
Week								
1	49.8	29.8	12	A	21.4	11.3	12	A
2	57.7	11.6	12	A	33.3	8.2	12	B
3	60.8	15.4	11	A	34.3	18.5	11	B

Note: *n* is the number of samples. SNK, grouping by the Student–Newman–Keuls test (arcsine-transformed data). Different letters indicate a significant difference at the $p < 0.05$ level.

Table 3. Mean percent recovery and SD of *Cryptosporidium* oocysts from raw waters for each filtration method and sample type (two-way ANOVA).

	Week 1 removed				All data			
Test	Mean	SD	*n*	SNK	Mean	SD	*n*	SNK
Method								
Flatbed filtration	26.7	28.4	12	A	35.6	26.4	18	A
Hemoflow	28.3	24.3	12	A	25.6	20.3	18	B
Envirochek™ HV	18.4	18.7	12	B	24.9	18.3	18	B
FiltaMax™	18.9	10.6	11	B	20.4	9.0	17	B
Sample type								
Sample A	—	—	—	—	33.6	14.8	24	A
Sample B	5.1	6.1	24	A	5.1	6.1	24	B
Sample C	42.0	14.2	23	B	42.0	14.2	23	C

Note: *n* is the number of samples. SNK, grouping by the Student–Newman–Keuls test (arcsine-transformed data). Different letters indicate a significant difference at the $p < 0.05$ level.

Cryptosporidium oocysts than the other 3 methods (35.6%) (Table 3). However, with the week 1 data removed, there was no significant difference between flatbed filtration and the Hemoflow method (recovery efficiencies of 26.7% and 28.3%, respectively). These recovery efficiencies were significantly higher than those obtained using the Envirochek™ HV and FiltaMax™ filtration methods (Table 3).

Table 4 shows the mean recovery efficiency for ColorSeed™ *Giardia* cysts for each filtration method. When all raw water data were included, there was no significant difference between the flatbed filtration, FiltaMax™, and Envirochek™ HV, and all were significantly higher than the Hemoflow method (47.8%, 41.4%, 40.6%, and 22.7%, respectively). With the week 1 data removed, flatbed filtration showed a significantly higher mean recovery of *Giardia*

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Table 4. Mean percent recovery and SD of *Giardia* cysts from raw waters for each filtration method and sample type (two-way ANOVA).

	Week 1 removed				All data			
Test	Mean	SD	*n*	SNK	Mean	SD	*n*	SNK
Method								
Flatbed filtration	42.2	12.1	12	A	47.8	14.6	18	A
FiltaMax™	29.0	7.5	11	B	41.4	18.6	17	A
Envirochek™ HV	29.3	9.4	12	B	40.6	18.7	18	A
Hemoflow	20.9	7.0	12	B	22.7	8.7	18	B
Sample type								
Sample A	—	—	—	—	53.2	18.6	24	A
Sample B	28.7	11.7	24	A	28.7	11.7	24	B
Sample C	32.1	12.0	23	A	32.1	12.0	23	B

Note: *n* is the number of samples. SNK, grouping by the Student–Newman–Keuls test (arcsine-transformed data). Different letters indicate a significant difference at the $p < 0.05$ level.

Table 5. Mean percent recovery and SD of EasySeed™ *Cryptosporidium* and *Giardia* (oo)cysts spiked before the IMS step for each method and sample type (two-way ANOVA).

	Cryptosporidium oocysts				*Giardia* cysts			
Test	Mean	SD	*n*	SNK	Mean	SD	*n*	SNK
Method								
FiltaMax™	93.9	6.7	16	A	93.1	10.6	16	A
Hemoflow	88.7	8.0	18	B	86.6	9.2	18	A
Flatbed filtration	86.6	9.8	18	B	85.4	21.7	18	A
Envirochek™ HV	84.5	10.2	19	B	87.0	7.1	19	A
Sample type								
Sample A	89.4	7.7	13	A	93.2	9.8	13	A
Sample B	87.7	8.9	12	A	93.4	16.9	12	A
Sample C	83.2	14.0	12	A	81.0	8.9	12	A
Deionized water	89.7	7.6	34	A	86.4	13.6	34	A

Note: *n* is the number of samples. SNK, grouping by the Student–Newman–Keuls test (arcsine-transformed data). Different letters indicate a significant difference at the $p < 0.05$ level.

cysts than the other 3 methods (42.2% compared with 29.0% for FiltaMax™, 29.3% for Envirochek™ HV, and 20.9% for Hemoflow).

Recoveries of EasySeed™ were consistently high (> 84%), indicating that few (oo)cysts were lost during IMS and immunofluorescent antibody – DAPI processing (Table 5). The FiltaMax™ method recovered a significantly higher number of *Cryptosporidium* oocysts compared with the other 3 methods (mean recovery of 93.9% compared with 84.5–88.7%); however, there was no significant difference between the recoveries obtained for *Giardia* cysts. The difference in oocyst recovery may be due to a lower proportion of materials that subsequently interfere with the *Cryptosporidium* IMS step being eluted during the concentration process.

Discussion

In this study, flatbed membrane filtration achieved the highest rate of recovery for *Giardia* cysts (42.2%, week 1 data removed) from raw waters with turbidity of up to 15 NTU. This agrees with the findings of du Preez et al. (2003), which indicated that their flatbed technique was capable of *Cryptosporidium* and *Giardia* recovery similar or superior to that of Envirochek™ and FiltaMax™ cartridges. Both flatbed filtration and Hemoflow ultrafiltration cartridges gave significantly higher recovery of *Cryptosporidium* oocysts from raw waters than either the Envirochek™ or the FiltaMax™ method (26.7% and 28.3% compared with 18.4% and 18.9%, respectively). The mean recovery efficiency for *Cryptosporidium* oocysts from raw waters using the Hemoflow ultrafiltration cartridge (28.3%) was slightly lower than the mean recovery efficiency reported by Simmons et al. (2001) of 42% for both reagent and surface water samples. Comparison of this study with that of Simmons et al. (2001) shows that the mean recovery efficiency for *Cryptosporidium* oocysts from raw waters using the Envirochek™ HV (18.4%) was lower than their mean recovery from surface waters (46%) but the recovery for deionized waters (64.4%) was higher than their recovery from reagent water (15%). Kuhn and Oshima (2002) reported higher mean recovery efficiencies for *Cryptosporidium parvum* oocysts using both ultrafiltration (74.1%) and the Envirochek™ (71.9%) when concentrating low-turbidity water samples (3.9 NTU). However, the mean recovery efficiency for *Cryptosporidium* oocysts using the ultrafiltration method with all types of surface waters was 47.9% (Kuhn et al. 2002), which is still higher than the Hemoflow recovery efficiency reported in this study (28.3%). Using a reusable ultrafiltration system and 10-L samples, Morales-Morales et al. (2003) reported mean *Cryptosporidium* recoveries of

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54% from a river water (n = 3) and of 32% from sites previously identified as problematic for oocyst recovery (n = 5). These recovery levels can be regarded as broadly similar to those observed in this study, although the small number of samples and varied water sources do not allow a conclusive comparison. The mean recoveries efficiency for *Cryptosporidium* oocysts and *Giardia* cysts using the FiltaMax™ method were 18.9% and 29.0%, respectively. In comparison, Sartory et al. (1998) reported *Cryptosporidium* recovery rates of 76.9–97.2% in partial-pellet analysis of FiltaMax™ eluates from a small number (n = 8) of 10- to 20-L raw water samples of turbidity 6–19 NTU, with recoveries dropping to 56.4–65.5% (n = 4) when the concentrates were processed using the flotation cleanup procedure then in use. Our mean recoveries for the FiltaMax™ were also lower than those reported in a recent study by McCuin and Clancy (2003) where recovery efficiency for *Cryptosporidium* and *Giardia* (oo)cysts from raw source water samples ranged from 19.5% to 54.5% for oocysts and from 46.7% to 70.0% for cysts.

The EasySeed™ spiking results showed consistently high recovery, demonstrating that losses of target organisms in the process steps following concentration–elution were small. This agrees with the findings of Feng et al. (2003). However, it is interesting that the FiltaMax™-processed pellets gave a significantly higher recovery of EasySeed™ *Cryptosporidium* oocysts (Table 5). This might suggest that the FiltaMax™ pellet contained less debris carried over from the raw water sample, producing a “cleaner” pellet.

Although flatbed membrane filtration had the highest recovery efficiency for *Giardia* cysts, while Hemoflow and flatbed filtration had the highest recovery of *Cryptosporidium* oocysts from raw waters, other methods tested may be preferable for certain applications. For example, the cartridge filtration methods (Envirochek™ HV and FiltaMax™) can be used for the collection of composite samples and facilitate on-site collection and concentration at remote sites, while ultrafiltration and flatbed membrane filtration have traditionally been laboratory-based methods used for grab samples. Ultrafiltration has been used for the concentration of viruses from water samples (Winona et al. 2001), with the potential for routine protozoological concentrations. Kfir et al. (1995) indicated that ultrafiltration could potentially be used for the simultaneous concentration of viruses, *Cryptosporidium* oocysts, and *Giardia* cysts from raw waters, while Morales-Morales et al. (2003) concluded that a reusable ultrafiltration system was ready for field use to simultaneously detect bacterial, viral, and protozoan pathogens. The availability of a low-cost, disposable ultrafiltration cartridge, such as the Hemoflow, means that it is now theoretically feasible to concentrate raw water samples for the simultaneous detection and enumeration of viral and protozoan pathogens without risk of cross-contamination between samples. The costs of consumables for the Hemoflow and flatbed methods were approx. one quarter of the costs for the Filtamax™ and Envirochek™ HV methods, while processing times for both the Envirochek™ HV and the flatbed methods were significantly less than for the other 2 methods.

The use of an internal quality control for the detection of *Cryptosporidium* and *Giardia* enabled the count from each water sample to be adjusted for the recovery efficiency of the concentration–elution process. The applicability of ColorSeed™ as an internal control in water samples has been discussed by Warnecke et al. (2003), validating its behaviour in comparison with nonmodified seed material and the robustness of the colour-labelling process. The standard use of such controls would greatly assist with the determination of accurate and reliable quantitative data regarding concentrations of *Cryptosporidium* and *Giardia* (oo)cysts in water, significantly improving confidence in the quality of such data and in the laboratory processes used to generate this data. Additionally, the use of precision inocula such as EasySeed™ and ColorSeed™ in method comparison studies enables more accurate determination of method performance than has previously been possible, again improving confidence in the resulting data.

It should be noted that the 13-mm membrane staining procedure used here is a deviation from standard staining methods (DWI 1999; USEPA 1999) using well slides. As briefly discussed by Warnecke et al. (2003), this procedure overcomes the losses of target organisms washing off the slide during staining (Reynolds et al. 1999) but precludes the use of differential interference contrast microscopy for subsequent identification of objects. Also varying from the standard methods was the increased flow rate used for the concentration of water samples with the FiltaMax™ cartridges. However, the flow rate used (4 L/min) was within the range recommended in the manufacturer’s instructions, so this variation would not be expected to have significantly impaired the subsequent elution of the target organisms.

In conclusion, all 4 concentration–elution methods examined here were capable of recovering *Cryptosporidium* oocysts and *Giardia* cysts from raw water samples at rates useful for monitoring purposes. Each method had advantages and limitations that may affect its use in different situations, such as compliance with local regulatory requirements, requirements for field sampling or continuous concentration, limitations of sample transport, ability to simultaneously examine for different organisms, financial costs, time requirements, and laboratory capabilities. The IMS, staining, and microscopy methods used were consistently efficient, with little loss of target organisms observed from these processes.

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