



Methods for the recovery, isolation and detection of *Cryptosporidium* oocysts in wastewaters

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Abstract

This correspondence describes the successful development of methods for the recovery, isolation and detection of *Cryptosporidium* oocysts in wastewater and biosolids. Wastewater from one plant was used to optimize methods in raw influent as well as primary, secondary and tertiary effluents. Raw influents and primary effluents were concentrated using centrifugation followed by isolation of *Cryptosporidium* oocysts using immunomagnetic separation (IMS) and detection of recovered organisms using epifluorescence microscopy. Mean oocyst recovery in raw influent was $29.2 \pm 12.8\%$ and $38.8 \pm 27.9\%$ in primary effluent at three sample volumes tested. Secondary and tertiary effluents were analyzed using a modified Method 1622 resulting in mean oocyst recoveries of $53.0 \pm 19.2\%$ and $67.8 \pm 4.4\%$, respectively. In biosolids with approximately 10% total solids, mean oocyst recovery was $43.9 \pm 10.1\%$ using IMS with a 5 g (wet weight) sample size. Due to the variability in these matrices, an internal microbiological standard was incorporated to serve as a tool for method performance.

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1. Introduction

Although waterborne cryptosporidiosis outbreaks occurred prior to the 1993 Milwaukee outbreak, it was this event that made the water industry recognize the challenge of controlling this protozoan parasite in drinking water. Although the Surface

Water Treatment Rule had been recently promulgated, it was clear that compliance with the Rule was not protective of public health with respect to *Cryptosporidium*. Monitoring of raw and finished drinking water for *Cryptosporidium* oocysts became a widespread practice. The early methods, including the USEPA Information Collection Rule method (USEPA, 1996) were poor from a number of aspects: 1) the filters used for sample collection were porous to oocysts and many passed through the filter fibers, 2) buoyant density gradient separation was used to separate the oocysts from other debris but is non-

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specific and many oocysts were lost in the clarification step, 3) uneven distribution of oocysts in samples coupled with analysis of small subsamples resulted in significant under- and overestimation of oocysts; the microscopic identification of oocysts is subjective and interpretations varied even among well trained analysts viewing the same slide (Clancy et al., 1999). Taken as a whole, the ICR method was characterized by high variability and false positive and false negative results were reported often. (Clancy et al., 1994; Nieminski et al., 1995).

In 1997, the USEPA undertook development of a new method for *Cryptosporidium* recovery and enumeration in water—Method 1622: *Cryptosporidium* in Water by Filtration/IMS/FA, was published (USEPA, 1997). Methods improvements included: 1) a new filter, allowing complete capture and high and reproducible elution of oocysts from the filter; 2) introduction of a new technique—immunomagnetic separation (IMS)—permitting specific capture of target organisms, and reducing false positives and background significantly; 3) an additional staining step to further aid in identification of *Cryptosporidium* oocysts; and 4) stringent quality assurance and quality control (QA/QC) as part of the method (Clancy et al., 1999). The USEPA conducted collaborative trials of the method to develop initial performance criteria (USEPA, 1999) and the method was used in the ICR Supplemental Survey to develop higher quality *Cryptosporidium* occurrence data (Connell et al., 2000). This multi-lab dataset was used to develop the acceptance criteria for method performance and these are defined in the latest version of the method (USEPA, 2001).

The methods available for monitoring *Cryptosporidium* in water were developed for water matrices, specifically source water and treated drinking water. No methods specifically suited for analysis of wastewaters were available. US researchers used adaptations of the ICR method, while UK researchers relied on variations of clinical methods for stool analysis for examination of wastewaters. In the UK, two methods were proposed for collection/concentration of wastewater influents/effluents in the Standing Committee of Analysts (SCA) blue book method (Anon, 1990). For wastewater effluents, one SCA method requires filtration of 10–50 L of wastewater effluents through a polypropylene car-

tridge filter at a flow rate of 1.5 L min^{-1} . The cartridge filters were processed according to the SCA method (Anon, 1990), which is very similar to the cartridge filter processing/concentration protocols described in the ICR method. Oocysts are clarified from contaminating debris by using a cold sucrose solution of a specified density and concentrated to a minimal volume. The second SCA method advocates screening of 5 L grab sample through a coarse (50–150 μm) filter and samples are concentrated by repeated centrifugation to 10 mL, subjected to sucrose density flotation and concentrated to a minimal volume (0.5–1.0 mL; depending upon the levels of contaminating debris). Both these procedures were derived or suggested approaches rather than protocols developed after experimental validation. However, data have indicated that the use of 100 L filtered samples yield consistently lower numbers of oocysts than 2 L grab samples concentrated by centrifugation alone (Robertson et al., 1995; Bukhari et al., 1997). These investigations also indicated that for some filtered samples it was necessary to perform two or three sucrose density flotation steps before samples were in an acceptable state for microscopy, contributing to significant loss of target organisms.

2. Objectives

The objective of this work was to develop standard methods for recovery, isolation and detection of *Cryptosporidium* oocysts in raw wastewater influents, primary, secondary and tertiary effluents, and biosolids. Wastewater from a tertiary treatment plant located in Saint Albans, VT was used for the development of these methods. The wastewater methods developed were based on modifications to the USEPA Method 1622. An internal positive control was included in the analysis of each sample to assess method performance. Initially, the internal control was seeded to wastewater samples that had also been seeded with viable oocysts. Statistical analysis of results was performed to determine if any differences were noted in volumes analyzed, difference in viable recoveries versus internal control recoveries and differences in recoveries using different IMS kits.

3. Materials and methods

3.1. *Cryptosporidium* oocyst spike dose source and enumeration

Cryptosporidium parvum oocysts were obtained from the Sterling Parasitology Research Laboratory, University of Arizona. This isolate was originally isolated from a calf and is referred to as the Iowa strain (Moon and Bemrick, 1981). It has been maintained by passage in neonatal calves. The feces of experimentally infected calves was collected and clarified by using cesium chloride density gradient centrifugation (Arrowood and Donaldson, 1996). Purified oocysts were stored in deionized water (DI) and antibiotics (gentamicin, 0.1 mg/mL; streptomycin, 0.1 mg/mL; and penicillin, 100 U/mL) at 4 °C. The age of oocysts used in these trials was less than three months.

Stock suspensions were diluted to a concentration of $1 \times 10^3 \text{ mL}^{-1}$ with DI and stored at 4 °C. Suspensions of *C. parvum* oocysts were enumerated as described in Method 1622 (USEPA, 2001). Briefly, ten 100 μL replicate aliquots were spotted onto individual wells of three-well treated microscope slides (Meridian Diagnostics, Inc., Cincinnati, OH, product no. R2206), dried at 42 °C for 1–2 h, fixed with absolute methanol, and air-dried for 3–5 min. Fluorescein isothiocyanate conjugated anti-*Cryptosporidium* sp. monoclonal antibodies (FITC-mAb; Waterborne, Inc. New Orleans, LA) were placed into each well. The slides were placed in a humid chamber and incubated at 35 °C for 45 min. Excess FITC-mAb was aspirated and each well was rinsed with 150 mM phosphate buffered saline (PBS), pH 7.2, and aspirated. A weak 4' 6-diamidino-2-phenylindole (DAPI) solution (0.4 $\mu\text{g mL}^{-1}$ in PBS) was placed into each well, incubated at room temperature for 2 min and excess DAPI solution was removed by aspiration. The wells were rinsed with PBS and then with DI water and dried in the dark at room temperature. Mounting medium (2% DABCO in 60% glycerol–40% PBS) was placed in the center of each well, a coverslip was applied, sealed with clear nail polish and slides were examined using epifluorescence microscopy. Spike doses were used within 24 h of enumeration.

3.2. ColorSeed™ flow-sorted spike dose suspensions

ColorSeed is a commercially available product from Biotechnology Frontiers Pty, Ltd (BTF, PO Box 599, North Ryde BC, NSW 1670, Australia). This product contains flow-sorted, gamma-irradiated *Cryptosporidium* oocysts and *Giardia* cysts that have been permanently stained with a red fluorescent dye. The standard deviation of cell counts of each organism for approved batches is less than 2.5. Batches are prepared and have a shelf life of 4 months if stored at 2–8 °C. ColorSeed was transferred to the test sample (or filter) using the method prescribed by the manufacturer. Briefly, 2 mL 0.05% Tween 80 was added to the contents of a ColorSeed vial. The vial was capped and shaken vigorously 25 times. The cap was removed and the contents were decanted to the test sample. A 3 mL volume of deionized water (DI) was added to the vial, the cap replaced and the tube was again vigorously shaken 25 times. The rinse was added to the test sample and the rinse step was repeated twice.

3.3. Method for recovery, isolation and detection of oocysts in raw influents and primary effluents

Raw influent and primary effluent grab samples (10 L) were collected on the day of analysis. In initial trials, triplicate subsamples (250 mL, 500 mL and/or 1 L) were seeded with approximately 100 viable *C. parvum* oocysts. In addition, a subsample of wastewater at each volume tested was analyzed unseeded to determine background concentration of oocysts in the sample. In later trials, ColorSeed was also added to each test volume using the transfer protocol described above. An appropriate volume of a 20% Tween 80 solution was added to each subsample to yield a final concentration of 1% and was then thoroughly mixed. Each sample was concentrated by centrifugation (1500 $\times g$; 15 min) in 250 mL conical centrifuge tubes (Corning Inc. Corning, NY part no. 430776), the pellet volume recorded and the supernatant aspirated to approximately 5–8 mL. *C. parvum* oocysts in the concentrates were isolated using either the Dynal or Aureon IMS kits as described below and recovered organisms were enumerated using epifluorescence microscopy.

3.4. Method for collection, recovery, isolation and detection of oocysts in secondary and tertiary effluents using HV filters

Pall Envirochek™ HV filters (Pall Corp. Ann Arbor, MI, product no. 12099) were used to capture and retain oocysts in secondary and tertiary effluents. During method development trials, spike doses of approximately 100 live *C. parvum* oocysts were seeded into triplicate 10 L volumes of secondary effluent to assess recoveries. In later trials *ColorSeed* was included in the trials to compare recoveries with live oocysts. Briefly, a seeded 10 L volume of secondary effluent was filtered through an HV filter at a flow rate of 2 L min⁻¹. For tertiary effluents, live oocysts were seeded directly into the capsule prior to sample filtration. The rationale for performing the seeding trials in this manner was due to the large sample volumes collected for the tertiary effluents. Adding the seed dose first offers the advantage of knowing that the entire dose is in the filter regardless of the volume filtered. In addition, applying the seed dose first creates a worst-case scenario in that the seed dose is underneath the sample. For each matrix tested an unseeded sample was also analyzed to determine background concentrations of the target organism.

A pre-elution step was performed for the secondary and tertiary effluents by adding a 5% (weight per volume) solution of sodium hexametaphosphate (Fisher Scientific, Inc. Pittsburgh, PA cat. no. S333) through the inlet port with a volume sufficient to cover the pleated membranes in the capsule. The capsule was placed on the wrist shaker (Lab-Line Instruments, Inc., Melrose Park, IL, Model no. 3589) and shaken at maximum speed for 5 min. The solution was pulled through the filter by applying a vacuum at the outlet port, so that re-dissolved material could exit through the filter pores, but oocysts and other intact particles greater than 1 µm would remain in the capsule. This action was followed by a DI rinse, which was poured into the inlet of the capsule and also pulled through using a vacuum, continuing the purge of dissolved or sub-micron material. These preliminary elution steps were followed by the standard Method 1622 procedure. Briefly, Laureth-12 buffer was added to the top of the pleats of the capsule. The filter was placed on the wrist shaker with the Luer-lock vent at the 12 o'clock position. The

filter was then shaken at maximum speed for 5 min. The filter wash was decanted to a 250 mL conical centrifuge tube. Fresh L-12 buffer was added above the pleats of the capsule and placed on the wrist shaker with the Luer-lock at the 4 o'clock position. The filter was then shaken for 5 min at full speed. Without decanting the filter wash, the filter was rotated so that the Luer-lock was at the 8 o'clock position and shaken for another 5 min. At this point, the filter wash was decanted to the 250 mL conical tube containing the initial sample eluant and was concentrated by centrifugation (1500 ×g; 15 min). The supernatant was aspirated to 5–8 mL and quantitatively transferred to a Leighton tube containing IMS buffers. *C. parvum* oocysts in the concentrates were isolated using either the Dynal or Aureon IMS kits as described below and recovered organisms were enumerated using epifluorescence microscopy.

4. Biosolids

4.1. Oocyst recoveries with the dynal IMS kit

Six replicate 5 g subsamples of St. Albans sludge were transferred into individual 30 mL glass beakers using 4–5 mL DI. Each sample was mixed gently to create a uniform suspension and then decanted into a Leighton tube. DI (2–3 mL) was added to each beaker to rinse the residual sludge suspension. One milliliter of each IMS buffer, SL-A and SL-B was added to each beaker and transferred to the respective Leighton tube. A predetermined number of *C. parvum* oocysts (~100) were added to each resuspended sludge sample and each sample was mixed by gentle inversion. One 5 g subsample of sludge was unseeded to determine the background levels of oocysts. Anti-*Cryptosporidium* IMS beads (100 µL) were added to each Leighton tube. The samples were allowed to rotate for 1 h and the bead–oocyst complexes were isolated from the supernatant by application of magnetic separation. The bead–oocyst complexes were resuspended in 10 mL of PBS to perform one additional rinse. Following this, the IMS procedure and detection using immunofluorescence microscopy were performed as previously described. To determine whether kaolin exerted a beneficial effect on oocyst recoveries from sludge samples, 11 replicate 5 g subsamples of

sludge were prepared for analysis as described above. Five of the seeded replicates and one unseeded replicate were processed using the protocol described above, whereas kaolin (0.75 g) was added to each of the five remaining seeded replicates. All samples were subjected to the IMS procedure and recovered oocysts were enumerated using immunofluorescence microscopy. Trials were also extended to evaluate the effect of the overall procedure by spiking the re-hydrated sludge in the 30 mL beaker with *C. parvum* oocysts and then transferring the spiked suspensions into Leighton tubes.

To determine if the IMS kit could recover seeded oocysts from a larger amount of biosolids without compromising oocyst recovery rates, a trial was performed by suspending 10 and 15 g of St. Albans sludge in 20 and 30 mL of PBST, respectively. Each suspension was seeded with ~100 oocysts, thoroughly mixed and then divided between two Leighton tubes. Seeded oocysts were recovered using IMS protocols with the following exceptions. Instead of adding 100 μ L of beads to each Leighton tube, 50 μ L of beads were used. The bead–oocyst complex was isolated using IMS, however when the complex was suspended in 1 mL SL-A buffer, both suspensions were pooled into a single Eppendorf tube for each sample.

4.2. Oocyst recoveries using density gradient flotation

Sludge weights equivalent to 0.5 g dried sludge were weighed into individual 50 mL conical tubes. Five replicates of each sludge type (St. Albans, ALCOSAN dewatered and lime-stabilized) and duplicate DI samples were processed. Each sample was diluted to 20 mL with PBST and seeded with approximately 100 *C. parvum* oocysts and thoroughly mixed by vortexing. This was followed by density gradient flotation on each sample followed by specific isolation of *C. parvum* oocysts using IMS. Recovered oocysts were enumerated using immunofluorescence microscopy.

4.3. Dynal IMS procedure

Each sample concentrate was transferred to an individual Leighton tube (LT) containing 1.0 mL each of SL-A and SL-B IMS buffers from a Dynal *Cryptosporidium* IMS kit (Dynal AS PO Box 158

Skøyen N-0212 Oslo, Norway, product no. 730.01). Each tube was then rinsed with 2 mL phosphate buffered saline containing 0.01% Tween 20 (PBST) and the rinse was transferred to a LT containing its respective sample. Regardless of the volume of raw influent or primary effluent being processed, 0.75 g kaolin (Sigma Chemical, Inc., St. Louis, MO catalog no. K7375) was added to each LT containing these concentrates. No kaolin was added to concentrates of secondary or tertiary effluents. To each tube, 100 μ L of the Crypto Dynabeads were added and incubated for 1 h while rotating on sample mixer (18 rpm) at room temperature. At the end of the incubation period, the beads were concentrated by placing the LT in the MPC-1 with the flat side of the LT facing the magnet. For raw wastewater and primary effluents, the tube was gently rocked through a 90° arc for 1 min and then allowed to stand undisturbed for 3 min. At the end of 3 min, the tube was gently rocked in the MPC-1 for 30 s. The supernatant was decanted and without removing the tube from the magnet, 10 mL PBS was added down the side of the tube opposite the beads. The tube was removed from the magnet and gently rocked five times to resuspend the beads and was then placed back in the MPC-1. The tube was gently rocked for 1 min and again allowed to stand undisturbed for 3 min. The tube was gently rocked for 30 s and the rinse was decanted. For secondary and tertiary effluents, the LT was gently rocked through a 90° arc for 2 min and the supernatant decanted. For all samples, the bead–(oo)cyst complex was resuspended in 1 mL of 1 \times SL™-buffer A and transferred to a 1.5 mL Eppendorf tube. The tube was placed in the MPC-M with the magnetic strip in place and was rocked 180° for 2 min to concentrate the bead complex at the back of the tube. The supernatant was discarded. The tube was removed from the MPC-M. The bead–oocyst complex was resuspended in 100 μ L 0.1N HCl, vortexed and incubated for 5 min at room temperature. After the incubation period, the tube was vortexed and placed in the MPC-M with the magnetic strip in place. The beads collected at the back of the tube and the acidified suspension was transferred to the well, of a three-welled slide, containing 10 μ L 1.0 N NaOH. After drying at 42 °C, the sample was

methanol-fixed and stained with Waterborne FITC-mAb and DAPI. The slides were examined using epifluorescence microscopy.

4.4. Aureon IMS procedure

Samples subjected to the Aureon IMS protocol were concentrated to approximately 5 mL. The pellet was vortexed and transferred to a Leighton tube (LT) for isolation of oocysts from the debris using IMS. A 5 mL rinse volume of IMS buffer 'A' was used to scour residual sample from the centrifuge tube and was transferred to the LT. For raw wastewater, kaolin (0.75 g) and *Cryptosporidium* A-Beads (100 μ L) were added and the tube was placed a sample rotator at 25 rpm for 45 min. For secondary and tertiary effluent samples, *Cryptosporidium* A-Beads (100 μ L) from the kit were added to the LT, tubes were capped and rotated at 25 rpm for 45 min. Samples were then removed from the mixer and inserted into the magnetic separation device (MagnetOn 4T). The magnet was gently rocked for 1 min and then returned to the upright position and allowed to stand undisturbed for 3 min. The magnet was then gently rocked for another 30 s and the supernatant was discarded. To resuspend the beads, 1 mL of A-Wash buffer was added and the tubes were gently mixed. Using a Pasteur pipette, the resuspended beads were transferred to a 2.0 mL cryovial with a rounded bottom. The vial was capped and placed in the MagnetOn 4T and rocked gently for 30 s. With the vial remaining in the magnetic device, the wash buffer was gently aspirated using a Pasteur pipette. Tubes were rinsed with a fresh 1 mL aliquot of A-Wash buffer, which was then transferred to the cryovial for another rinse. The bead separation procedure was repeated. Fifty microliters of 0.05 N HCl was then added to the cryovial and vortexed for 15 s. The vial was allowed to stand for 30 s and then vortexed for an additional 15 s. The cryovial was placed in the MagnetOn 4T and allowed to stand for 10 s. The IMS concentrate was transferred to a well slide. The acid disassociation step was repeated with the second acidic suspension being placed in the same well as the first. The well was placed on the slide warmer to dry. Recovered oocysts were stained and enumerated as previously described.

4.5. Epifluorescence microscopy

A Zeiss Axioskop fluorescence microscope, equipped with a blue filter block (excitation wavelength, 490 nm; emission wavelength, 510 nm) was used to detect FITC-mAb labeled oocysts at a magnification of 360 \times . DAPI staining characteristics were observed at 640 \times magnification using a UV filter block (excitation wavelength, 400 nm; emission wavelength, 420 nm). A green filter block (excitation wavelength, 546 nm; emission wavelength, LP590) was used for visualization of the Texas Red stain of the *ColorSeed* at 640 \times magnification. Internal morphology of oocysts was observed by using Nomarski DIC microscopy at 640–1600 \times magnification.

4.6. Sample turbidity measurements

The turbidity of each unspiked sample matrix was measured prior to processing. A Hach 2100P turbidimeter, was used to determine turbidities. This turbidimeter is capable of measuring turbidity levels between 0 and 1000 ntu. If necessary, subsamples of the original matrix were diluted in DI to enable turbidity measurements. The appropriate dilution factor was used to estimate turbidity of the original sample. Packed pellet volumes of the unspiked matrices were recorded after centrifugation.

4.7. Statistical analyses

Statistical analyses were performed using Sigma StatTM software by Jandel Scientific. Analytic comparisons to assess the significance of differences in mean recovery rates were determined according to one way analysis of variance (ANOVA), based on the null hypothesis of equal sample means (recovery rates). In the case that ANOVA tests indicated inequality of sample means across the range of test conditions, further pair wise analyses were performed under a Student–Newman–Keuls multiple comparisons test. In the case that initial assessment of equality of variance in the mean recovery rates failed, Rank Sum tests (Mann–Whitney) were applied. In the case that the initial assessment of normality surrounding the sample distributions failed, nonparametric tests (Kruskal–Wallis analysis

of variance on ranks) were substituted for the ANOVA approach.

5. Results

5.1. Raw influent

Recovery data from the comparison of *ColorSeed* and live oocysts in raw wastewater are presented in Table 1. Live oocyst recoveries in 250 mL raw wastewater ranged from 2.7% to 79.9% using the Dynal IMS kit ($n=27$), with a mean recovery of $33.0 \pm 21.1\%$. When the volume of raw wastewater analyzed was increased to 500 mL, mean live oocyst recovery dropped slightly to $31.8 \pm 20.6\%$ ($n=21$).

In 1000 mL sample volumes mean recovery of live oocysts was $24.3 \pm 19.9\%$ ($n=21$) with the Dynal IMS kit. Initial trials incorporating *ColorSeed* in 250 mL raw influent yielded mean recoveries of $39.9 \pm 5.9\%$ for live oocysts compared to $35.7 \pm 7.7\%$ for *ColorSeed* oocysts (Table 1, trial 7). Further trials in raw wastewater comparing live versus *ColorSeed* recoveries showed a marked decrease in oocysts recoveries for both types of oocysts (Table 1, trials 8 and 9). However, in each trial, the *ColorSeed* recovery rate was similar to the rate of recovery of live oocysts, suggesting that *ColorSeed* could be a useful tool in determining overall method performance. In raw wastewater trials using the Dynal IMS kit and *ColorSeed* oocysts, mean *ColorSeed* recoveries were $23.5 \pm 13.8\%$ for

Table 1
Recovery rates of live *Cryptosporidium* oocysts and *ColorSeed* in raw wastewater

Trial	IMS kit (# replicates)	Vol. analyzed (L)	Packed pellet vol. (mL)	Spike dose live oocysts (mean \pm SD)	Percent recovery live oocysts (mean \pm SD)	Percent recovery <i>ColorSeed</i> (mean \pm SD)
1	Dynal (3)	0.25	0.25	101.4 \pm 9.2	71.0 \pm 10.0	NA
	Dynal (3)	0.50	0.5		38.1 \pm 9.4	NA
2	Dynal (3)	0.50	0.5	104.8 \pm 8.5	47.2 \pm 12.6	NA
3	Dynal (3)	1.00	0.5	110.6 \pm 9.8	48.8 \pm 20.1	NA
4	Dynal (3)	0.50	0.5	101.0 \pm 14.1	46.5 \pm 15.6	NA
	Dynal (3)	1.00	1.0		37.6 \pm 8.4	NA
5	Dynal (6)	0.25	1.0	101.1 \pm 4.9	42.6 \pm 14.4	NA
	Dynal (3)	1.00	4.0		21.4 \pm 18.4	NA
6	Dynal (3)	0.25	0.1	103.1 \pm 4.8	39.1 \pm 12.2	NA
	Dynal (3)	0.50	0.2		39.8 \pm 3.5	NA
	Dynal (3)	1.00	0.25		33.9 \pm 17.8	NA
7	Dynal (3)	0.25	0.2	90.3 \pm 8.0	39.9 \pm 5.9	35.7 \pm 7.7 ^a
8	Dynal (3)	0.25	0.25	111.1 \pm 12.3	5.7 \pm 4.4	7.4 \pm 1.5 ^a
	Dynal (3)	0.50	0.5		0.9 \pm 0.9	1.0 \pm 1.0 ^a
	Dynal (3)	1.0	1.0		1.2 \pm 2.0	0 \pm 0 ^a
9	Dynal (3)	0.25	0.2	105.6 \pm 8.3	19.9 \pm 9.1	9.2 \pm 6.1 ^b
	Dynal (3)	0.50	0.4		4.7 \pm 6.6	2.7 \pm 3.8 ^b
	Dynal (3)	1.0	0.8		2.5 \pm 1.1	0.7 \pm 0.6 ^b
10	Dynal (3)	0.25	0.25	96.0 \pm 7.4	17.7 \pm 1.8	37.1 \pm 3.1 ^b
	Aureon (3)	0.25	0.25		15.7 \pm 5.4	25.2 \pm 3.2 ^b
11	Aureon (3)	0.25	1.0	125.4 \pm 14.2	18.9 \pm 4.6	20.7 \pm 4.8 ^c
	Aureon (3)	0.50	2.0		7.2 \pm 2.8	6.8 \pm 6.2 ^c
	Aureon (3)	1.0	4.0		1.9 \pm 1.2	2.0 \pm 2.7 ^c
12	Dynal (3)	0.25	0.2	113.1 \pm 11.2	26.8 \pm 2.1	27.9 \pm 2.1 ^c
	Dynal (3)	0.5	0.4		45.4 \pm 7.7	50.7 \pm 3.6 ^c
	Dynal (3)	1.0	0.8		24.4 \pm 2.0	26.5 \pm 6.2 ^c

No indigenous oocysts were detected in unseeded samples in each trial.

^a *ColorSeed* spike dose concentration: 99 ± 1.2 *C. parvum* oocysts.

^b *ColorSeed* spike dose concentration: 98 ± 1.7 *C. parvum* oocysts.

^c *ColorSeed* spike dose concentration: 98 ± 1.4 *C. parvum* oocysts.

250 mL volumes of raw wastewater, $18.1 \pm 24.6\%$ for 500 mL volumes, and $9.1 \pm 13.5\%$ for 1000 mL volumes. Mean recoveries of live oocysts from raw wastewater using the Dynal product were $22.0 \pm 12.5\%$ for 250 mL, $17.0 \pm 22.0\%$ for 500 mL, and $9.5 \pm 11.3\%$ in 1000 mL [Table 1, trials 7, 8, 9, 10 (Dynal data only) and 12]. A Kruskal–Wallis One Way Analysis of Variance on Ranks revealed significant differences among the test groups ($p=0.039$); however, when multiple pair wise comparisons (Dunn's Method) were made these differences were not apparent and were most likely due to random sampling variability.

During the method development trials, Aureon Biosystems released an IMS kit for the isolation of oocysts from environmental matrices. Validation trials conducted in source waters in our laboratory yielded results that met the acceptance criteria established for Method 1623 (USEPA, 2001). We were curious to determine if this kit could perform as well as the Dynal kit in wastewater. In head-to-head comparison trials in 250 mL raw wastewater, live oocysts recoveries were $17.7 \pm 1.8\%$ using Dynal kits and $15.7 \pm 5.4\%$ using Aureon kits (Table 1, trial 10). Mean recoveries of *ColorSeed* in these trials were $37.1 \pm 3.1\%$ and $25.2 \pm 3.2\%$ for Dynal and Aureon, respectively. In this trial the *ColorSeed* mean recoveries were greater than the live mean recoveries by 20% for Dynal and 10% for Aureon. All trials conducted using the Aureon IMS kit yielded mean recoveries of live oocysts of $17.3 \pm 4.8\%$ ($n=6$), $7.2 \pm 2.8\%$ ($n=3$) and $1.9 \pm 1.2\%$ ($n=3$) in 250, 500 and 1000 mL sample volumes of raw wastewater, respectively (Table 1, trials 10 and 11). Mean recoveries of *ColorSeed* were $23.0 \pm 4.4\%$ in 250 mL, $6.8 \pm 6.2\%$ in 500 mL and $2.0 \pm 2.7\%$ in 1000 mL of raw wastewater.

When 250 mL volumes of seeded, raw wastewater were examined across all experimental conditions, significant differences in the recovery rates of live oocysts or *ColorSeed* using Dynal or Aureon were not observed ($p=0.063$). However, in one trial (trial 10) where 250 mL volumes of raw wastewater were analyzed, significant differences were noted in the recovery rates of *ColorSeed* using Dynal versus Aureon ($p=0.0004$). In these trials *ColorSeed* recoveries were notably higher than recoveries of live oocysts. Significant differences were not observed

with the 500 mL raw wastewater data set ($p=0.066$) when comparing live oocyst recoveries and *ColorSeed* recoveries using Dynal or Aureon IMS kits. However, significant differences ($p=0.027$) were revealed in the mean recoveries between these same test groups using 1000 mL sample volumes of raw wastewater. Statistical tests of all raw wastewater recovery data using the Dynal product failed to unearth significant differences in recovery of live oocysts as a function of sample volume ($p=0.251$). For the Aureon product, however, viable oocyst recoveries from raw wastewater were significantly different as a function of sample volume ($p=0.0007$), with recoveries decreasing as a function of increasing volume.

5.2. Primary effluent

Recovery data in primary effluent are presented in Table 2. Using the Dynal IMS kit, oocyst recoveries in primary effluent were slightly higher for both live and *ColorSeed* oocysts than in raw wastewater. In 250 mL sample volumes, live oocyst recoveries ranged from 10.9% to 107% and *ColorSeed* oocyst recoveries from 24.5% to 52%. Mean recoveries in 250 mL sample volumes were $42.7 \pm 28.3\%$ for live oocysts ($n=15$) and $36.2 \pm 11.4\%$ for *ColorSeed* oocysts ($n=6$). The mean live oocyst recovery in 500 mL sample volumes was similar to that observed in 250 mL at $42.8 \pm 31.5\%$ ($n=15$) while the mean *ColorSeed* oocyst recovery was slightly higher at $41.3 \pm 14.7\%$ ($n=6$). Mean oocyst recoveries in 1000 mL of primary effluent was $30.9 \pm 23.6\%$ (live, $n=15$) and $43.2 \pm 16.6\%$ (*ColorSeed*, $n=6$). No recovery trials of oocysts from primary effluent using the Aureon IMS kit were performed.

In trial 2, it was noted by plant personnel that chemical toilet waste from a passenger train had been dumped into the waste stream at some point prior to the sampling event. The mean concentration in the background samples was 73.3 ± 37.2 oocysts/L with oocyst concentrations ranging from 32/L in the 1000 mL subsample volume analyzed to 104/L in the 500 mL sample volume analyzed (52 oocysts/500 mL). Based on these analyses of the unseeded samples, the oocyst recoveries in this trial may be artificially high due to the high variability observed in the background oocyst concentration. ANOVA

Table 2
A comparison of recoveries in primary effluent using live *Cryptosporidium* oocysts and *ColorSeed*

Trial	Vol. analyzed (L) (# replicates)	Packed pellet vol. (mL)	Turbidity (ntu)	Spike dose live oocysts (mean \pm SD)	Percent recovery live oocysts (mean \pm SD)	Percent recovery <i>ColorSeed</i> (mean \pm SD)
1	0.25 (3)	0.1	41.3	95.3 \pm 11.6	41.3 \pm 17.0	NA
	0.50 (3)	0.15			15.0 \pm 8.9	NA
	1.0 (3)	0.25			2.8 \pm 2.2	NA
2	0.25 (3)	0.5	163	94.4 \pm 10.8	90.0 \pm 17.5 ^a	NA
	0.50 (3)	1.0			97.8 \pm 15.9 ^a	NA
	1.0 (3)	2.0			66.0 \pm 11.6 ^a	NA
3	0.25 (3)	0.1	77.7	112.6 \pm 6.5	40.0 \pm 2.3	NA
	0.50 (3)	0.2			32.6 \pm 8.0	NA
	1.0 (3)	0.5			12.4 \pm 0.9	NA
4	0.25 (3)	0.1	40.3	128.4 \pm 19.4	14.5 \pm 6.3	31.6 \pm 7.2 ^b
	0.50 (3)	0.2			23.4 \pm 9.6	41.8 \pm 22.1 ^b
	1.0 (3)	0.4			39.5 \pm 6.3	56.8 \pm 6.8 ^b
5	0.25 (3)	0.1	39.0	105.2 \pm 5.6	27.6 \pm 8.1	40.8 \pm 14.4 ^b
	0.50 (3)	0.2			45.0 \pm 5.7	40.8 \pm 7.2 ^b
	1.0 (3)	0.4			33.9 \pm 6.9	29.6 \pm 9.1 ^b

No oocysts detected in unseeded subsamples analyzed in each trial unless noted otherwise.

^a Concentration of *C. parvum* oocysts in background samples were as follows: 21 oocysts in 250 mL, 52 oocysts in 500 mL and 32 oocysts in 1000 mL sample.

^b *ColorSeed* spike dose concentration: 98 \pm 1.0 *C. parvum* oocysts.

analysis of all primary effluent data revealed no significant differences in live oocyst versus *ColorSeed* mean recoveries ($p=0.775$) regardless of the volume processed. Therefore, increasing the primary effluent volume analyzed to 1 L did not negatively impact oocyst recoveries and in fact, no discernable differences in mean oocyst recoveries were noted when a statistical analysis of the data set was performed.

5.3. Secondary and tertiary effluents

Recovery data for secondary and tertiary effluents are presented in Table 3. All samples were collected using the Pall Gelman Envirochek HV capsule filter incorporating sodium hexametaphosphate as a pre-treatment buffer for dissolving the material collected on the membrane surface followed by a DI rinse. Following this pretreatment, the retained oocysts were eluted from the filter and isolated from the interfering debris using IMS as described in Method 1622.

Differences in live oocyst recoveries using the two IMS kits were most prevalent in the secondary and

tertiary effluents. Mean live oocyst recovery in secondary effluent was 53.0 \pm 19.2% ($n=11$) with recoveries ranging from 21.9% to 75.2% using the Dynal IMS kit. Live oocyst recoveries using the Aureon IMS kit ranged from 8.8% to 10.4% ($n=3$) with a mean of 9.3 \pm 0.9%. Mean *ColorSeed* oocyst recoveries in secondary effluent were 30.4 \pm 17.5% ($n=6$) using Dynal IMS kit compared to 3.4 \pm 0.6% ($n=3$) using the Aureon kit. ANOVA tests revealed significant differences ($p=0.0003$) in this comparison of recovery rates. Multiple comparisons analysis demonstrated the most significant differences in live oocyst recoveries using Dynal versus Aureon, with a difference of the mean recoveries of 43.6% as well as recovery rates for *ColorSeed* versus live oocysts using Dynal.

A mean recovery of 67.8 \pm 4.4% ($n=3$) was achieved using Dynal compared to 41.2 \pm 13.8% ($n=9$) with Aureon in isolating live oocysts from tertiary effluent. Significant differences were noted ($p=0.0014$) in the recovery of live oocysts using the two products with differences in mean recoveries of 26.6%. *ColorSeed* was recovered at a rate of 48.6 \pm 12.3% ($n=3$) and 28.1 \pm 13.5% ($n=9$) using

Table 3

Oocyst recoveries in secondary and tertiary effluents using live *Cryptosporidium* and *ColorSeed*

Matrix	IMS kit (# replicates)	Volume analyzed (L)	Packed pellet vol. (mL)	Turbidity (ntu)	Spike dose live oocysts (mean \pm SD)	% Recovery live oocysts (mean \pm SD)	% Recovery <i>ColorSeed</i> (mean \pm SD)
Secondary effluent	Dynal (2)	10	0.5	2.28	91.0 \pm 7.6	32.8 \pm 15.5	NA
	Dynal (3)	10	0.2	1.99	113.0 \pm 16.3	54.3 \pm 14.3	18.7 \pm 16.5 ^b
	Dynal (3)	10	0.1–0.7	3.21	112.6 \pm 6.5	46.8 \pm 23.1	NA
	Aureon (3)	10	0.1	2.90	125.4 \pm 14.2	9.3 \pm 0.9	3.4 \pm 0.6 ^c
	Dynal (3)	10	0.1	2.34	113.1 \pm 11.2	71.3 \pm 3.4	42.2 \pm 8.9 ^c
Tertiary effluent	Aureon (3)	75.7	0.05	– ^a	125.4 \pm 14.2	57.7 \pm 5.6	44.6 \pm 8.7 ^d
	Aureon (3)	62.0	0.05	– ^a	115.2 \pm 5.9	38.2 \pm 3.5	21.1 \pm 5.0 ^d
	Aureon (3)	52.0	0.05	– ^a	128.4 \pm 19.4	27.8 \pm 5.3	18.7 \pm 4.2 ^d
	Dynal (3)	39.2	0.05	– ^a	113.1 \pm 11.2	67.8 \pm 4.4	48.6 \pm 12.3 ^e

No oocysts detected in unseeded samples in each trial conducted.

^a Turbidity of tertiary effluent is approximately 2 ntu.^b *ColorSeed* spike dose concentration: 99 \pm 1.2 *C. parvum* oocysts.^c *ColorSeed* spike dose concentration: 98 \pm 1.7 *C. parvum* oocysts.^d *ColorSeed* spike dose concentration: 98 \pm 1.0 *C. parvum* oocysts.^e *ColorSeed* spike dose concentration: 98 \pm 1.4 *C. parvum* oocysts.

Dynal and Aureon, respectively with no significant differences noted.

6. Biosolids

In trials using 5 g of St. Albans digester sludge, with approximately 10% solids, direct IMS yielded oocyst recoveries ranging from 21.3% to 52.1% with a spike dose of approximately 100 organisms (Table 4, trials 1, 2, and 3a). The mean oocyst recoveries for these trials were 43.6 \pm 10.1%. Using

0.75 g kaolin in wastewater sludge the mean oocyst recovery was 30.8 \pm 8.3% (Table 4, trial 3b). When the sample size of the biosolids was increased to 10 or 15 g and analyzing as two subsamples, mean oocyst recoveries decreased to 21.1 \pm 5.4% and 7.6 \pm 4.3%, respectively. (Table 4, trials 4 and 5).

Using lime-stabilized samples from the Allegheny County Sanitary Authority (ALCOSAN) with considerably higher solid content (25–27%), it was difficult to get good mixing in the Leighton tubes. As a result, the Dynal mixing procedure was modified to utilize a magnetic panning approach using a tilting orbital platform shaker. Despite efforts to improve mixing, the oocyst recoveries in both dewatered and lime-stabilized sludge were less than 2% (data not shown). In contrast, where St. Albans sludge was used with this mixing protocol, mean oocyst recoveries were 42.5% indicating that the mixing procedure was effective and the poor recoveries in the dewatered and lime-stabilized sludge were probably associated with the solids content of sample matrix.

In order to reduce further the solids content in samples, their partial purification prior to IMS was investigated by using flotation procedures (sp. gr. = 1.10). A reduction in oocyst recoveries was noted in St. Albans sludge compared to direct IMS on 5 g samples (Table 5); however, where 2 g of dewatered sludge samples were used, oocyst recoveries improved

Table 4

Recovery of *C. parvum* oocysts from St. Albans sludge using immunomagnetic separation

Trial	Wet weight of sludge analyzed (g)	Spike dose (mean \pm SD)	Percent recovery (mean \pm SD)
1 (n=5)	5	93.9 \pm 8.0	41.1 \pm 9.2
2 (n=5)	5	94.5 \pm 10.1	48.3 \pm 11.4
3a (n=5)	5	104.0 \pm 12.3	43.1 \pm 5.6
3b (n=5)	5	104.0 \pm 12.3	30.8 \pm 8.3
4 (n=6)	10	88.8 \pm 8.5	21.1 \pm 5.4
5 (n=6)	15	88.8 \pm 8.5	7.6 \pm 4.3

Trial 1: *C. parvum* oocysts spiked directly into Leighton tubes. In remaining trials oocysts in spiked into samples in individual glass beakers. In trial 3b, 0.75 g kaolin was added to each replicate. No oocysts detected in unseeded subsamples analyzed.

Table 5
Oocyst recovery in sludge using density gradient flotation followed by IMS

Sludge	Weight analyzed (g)	<i>C. parvum</i> oocyst spike dose (mean \pm SD)	Percent recovery (mean \pm SD)
DI ($n=2$)	–	88.8 \pm 9.3	64.8 \pm 4.0
St. Albans ($n=5$)	5	88.8 \pm 9.3	25.9 \pm 6.1
ALCOSAN dewatered ($n=5$)	2	81.7 \pm 8.5	35.7 \pm 10.0
ALCOSAN lime-stabilized ($n=5$)	2	81.7 \pm 8.5	0.0 \pm 0.0 ^a

No oocysts detected in unseeded subsamples analyzed for each matrix.

^a A gelatinous floc formed during the flotation procedure.

to 35.7%. In contrast, the lime-stabilized sludge did not demonstrate improvements in recoveries by this procedure.

7. Statistical summary

Table 6 represents a statistical summary of comparisons made with oocyst recoveries with respect to increasing sample volume, different IMS kits, and live oocysts versus *ColorSeed*. The recovery of the *ColorSeed* oocysts was compared to the recovery of

seeded live oocysts in raw wastewater since live oocysts were initially used to assess method performance. With the limited trials conducted at that time, the recovery of *ColorSeed* oocysts seemed to provide a conservative estimate of how well the method was able to recover seeded live oocysts. More data on the recovery of *ColorSeed* oocysts and seeded live oocysts were generated in each of the wastewater matrices. In tertiary effluent, there were no significant differences between the recovery rates of *ColorSeed* oocysts and live oocysts according to Analysis of Variance (ANOVA) testing performed on mean oocyst recoveries ($p > 0.05$). The same conclusion was noted in trials conducted with primary and secondary effluents. In one trial with 250 mL sample volumes of raw wastewater, differences in mean *ColorSeed* recoveries were statistically significant (distinguishable) from the recovery of live oocysts ($p = 0.0004$). However, when a comparison of *ColorSeed* and live oocyst recoveries was made using all data from experiments with 250 mL sample volumes of raw wastewater, significant differences were not observed ($p = 0.0617$). In addition, no statistical differences were noted between *ColorSeed* and live oocyst recoveries in 500 and 1000 mL sample volumes of raw wastewater.

Direct comparisons of oocyst recoveries using Dynal and Aureon IMS kits were conducted in experiments with seeded raw wastewater and secon-

Table 6
Statistical analysis summary of wastewater methods comparisons

Condition	Test	Significant?	<i>p</i> value	Multiple pairwise comparisons
<i>Raw wastewater</i>				
250 \times 500 \times 1000 ^a	ANOVA	No	0.251	250 = 500 = 1000
250 \times 500 \times 1000 ^b	ANOVA	Yes	0.0008	250 \neq 500 = 1000
<i>Primary effluent</i> ^c				
250 \times 500 \times 1000 \times Live \times <i>ColorSeed</i>	ANOVA	No	0.775	250 = 500 = 1000—live or <i>ColorSeed</i>
<i>Secondary effluent</i>				
Dynal \times Aureon \times Live \times <i>ColorSeed</i>	ANOVA	Yes	0.0003	Dynal \neq Aureon—live
<i>Tertiary effluent</i>				
Dynal \times Aureon \times Live \times <i>ColorSeed</i>	ANOVA	Yes	0.0014	Dynal \neq Aureon—live

^a Using oocysts and Dynal IMS kit.

^b Using live oocysts with Aureon IMS kit.

^c Using Dynal IMS kit.

dary and tertiary effluents. The results of ANOVA analyses suggest that differences observed in the recovery efficiencies of both IMS products was greater than that expected by chance alone when live oocysts were seeded into tertiary and secondary effluents ($p=0.0003$ and $p=0.0014$, respectively); oocyst recoveries were greater with the Dynal IMS kit in both of these test matrices. The mean live oocyst recovery in secondary effluent using the Dynal IMS kit was $53.0 \pm 19.2\%$ compared to $9.3 \pm 0.9\%$ using the Aureon IMS kit. Mean live oocyst recoveries in tertiary effluent was $67.8 \pm 4.4\%$ and $41.2 \pm 13.8\%$ using Dynal and Aureon IMS kits, respectively. However, significant differences in recovery efficiencies by the two products were not observed when *ColorSeed* oocysts were seeded into these test matrices.

As seeded, raw wastewater sample volumes were increased from 250 to 1000 mL, differences in recovery efficiencies of live oocysts using the Dynal kit were insignificant ($p=0.251$). However, when the Aureon IMS kit was applied against seeded, raw wastewater samples, a decline in recovery efficiency was observed as sample volume increased to 1000 mL ($p=0.0008$). When raw wastewater was seeded with *ColorSeed* oocysts, recoveries using the two IMS products were not significantly different regardless of the sample volume analyzed. However, in pair wise comparisons of 250 mL sample volumes, a significant difference in *ColorSeed* oocyst recoveries was observed, where Dynal IMS kits achieved $32.5 \pm 5.6\%$ oocyst recovery and Aureon IMS kits achieved $23.0 \pm 4.4\%$ recoveries ($p < 0.05$). Based on the statistical analyses performed on each wastewater data set it appears the Dynal IMS kit offers improved performance under most test conditions. Comparisons of Aureon and Dynal recoveries using live oocysts and *ColorSeed* oocysts in seeded raw wastewater indicate no significant differences for the 250 and 500 mL sample volumes. ANOVA tests revealed that mean recoveries using these two products were not the same for the 1000 mL sample volumes, but it was not possible to discern pair wise differences under multiple comparisons analyses following the ANOVA tests on equal sample means. As volumes of raw wastewater analyzed were increased from 250 to 1000 mL, no significant differences in live oocyst recoveries were observed using Dynal while live oocyst recov-

eries with Aureon were adversely affected as the sample volume increased, with significant differences noted.

8. Discussion

Most published information to date on occurrence of *Cryptosporidium* in wastewater has been generated using modified versions of the ICR method in the US or the SCA method in the UK. Medema and Schijven (2001) reported concentrations of oocysts from <1 to $3.9 \times 10^5 \text{ L}^{-1}$ in settled raw influents. However, when recoveries were assessed using their modified ICR method they reported a mean oocyst recovery rate of 0.4%. Other studies conducted on *Cryptosporidium* occurrence in wastewater using the ICR or SCA method did not include an assessment of oocyst recovery rates (Chauret et al., 1999; Dumoutier and Mandra, 1996; Carraro et al., 2000; Gibson et al., 1998).

Due to the high concentration of particles and the complex nature of the wastewater matrices, some researchers have used alternate density gradients for clarification and isolation of oocysts from other debris. These procedures offer nonselective isolation of biological particles and may yield preparations containing high levels of contaminating debris that may occlude target organisms in the detection phase of the assay. Using a spike dose of approximately 120 oocysts/L, Robertson et al. (2000) achieved a mean oocyst recovery of 81% in 50–100 mL volumes of wastewater influents. Subsequent clarification techniques using ether, cold sucrose (1.18 sp. gr.) or combined ether/cold sucrose, yielded reductions in the mean oocyst recoveries to approximately 32%. Using 50 mL to 2 L grab samples, other researchers have used the centrifugation/clarification approach to recovery of oocysts in wastewater influents; however, recovery rates for methods used were not assessed (Bukhari et al., 1997; Payment et al., 2001; Quintez-Diaz et al., 2001; Gibson et al., 1998; Dumoutier and Mandra, 1996).

With the development of an IMS kit for the specific isolation of *Cryptosporidium* oocysts from water in the mid-1990s coupled with the improvements in the capture of oocysts in source water, the research team evaluated these novel techniques in the search for a

new and improved method for wastewater. The matrix composition of raw influents and primary effluents are such that filtration to concentrate the sample is impossible. Since Robertson et al. (2000) found that direct examination of small volumes of raw influents yielded high oocyst recoveries our initial trials repeated this work. In addition, concentration of oocysts in these small volumes (50 mL and 2 L) was followed by the specific isolation of *Cryptosporidium* using IMS. Mean recovery rates observed in these trials did not approach those obtained by Robertson et al. (2000). Mean oocyst recovery rates in raw influents and primary effluents were 17.0–21.1% when the concentrate was examined directly and rose to between 36.7% and 43.8% when the concentrate was subjected to IMS (data not shown). While direct examination of 2 L sample concentrates yielded recoveries similar to those achieved in 50 mL sample volumes, approximately 22%, subjecting these concentrates to IMS did not improve mean recovery rates, and dropped to 15.6%.

The data from these initial trials indicated inhibition of the IMS procedure in recovery of oocysts from raw wastewater concentrates; even when minimal grab sample volumes were analyzed. The separation procedure for oocysts from the suspending matrix was considered to be a critical component of the overall methodology and was expected to be the limiting factor with respect to the sample volumes that are likely to be collected. Examination of sodium chloride or sucrose density flotation from wastewater concentrates yielded poor and highly variable recoveries (data not shown). Furthermore, there was considerable carry-over of debris. This suggested that the use of IMS technology was the only viable option for efficient oocyst recovery and the procedure required optimization for raw wastewater concentrates. It was hypothesized that the inhibitory effects of large insoluble particles on IMS recovery efficiencies may be overcome by addition of a suitable buffer or chemical additive and, to this end, a chemical additive was sought.

During these investigations, the ASTM D5905-96 (Anon, 1998) defined substitute wastewater was prepared and evaluated for suitability as a standard matrix for use in *Cryptosporidium* method optimization studies. In these initial trials, oocyst recoveries in the artificial matrix exceeded 50% in the 50 mL or 2 L

grab samples by IMS. This prompted us to examine the constituents of the artificial matrix individually to determine possible inhibitory relief for the IMS. One constituent was kaolin or aluminum silicate. Raw influent concentrates generated from 250 mL sample volumes were seeded with approximately 100 oocysts and subjected to IMS with 0.1 and 0.5 g kaolin as well as no kaolin. Mean oocyst recoveries rose from $39.5 \pm 9.8\%$ with no kaolin to $60.9 \pm 4.9\%$ in the presence of 0.1 g kaolin and even higher with 0.5 g kaolin at $72.6 \pm 5.6\%$ (data not shown). It was hypothesized that the addition of kaolin sequestered interfering particles thereby exposing oocyst surface epitopes and making them more available for capture by the IMS beads. Although addition of kaolin had a beneficial effect with respect to capture of oocysts from the raw wastewater matrix, considerable quantities of kaolin were also carried over to the final sample concentrate. This obstacle was overcome by repeated rinsing of the bead–oocyst complex to remove excess wastewater and kaolin particles, prior to oocyst dissociation and detection. Repeating this simple procedure two–three additional times removes contaminating debris sufficiently to improve reliability of detection using immunofluorescence and Nomarski-DIC microscopy. This modification helped to yield significantly reduced number of interfering particles in the bead–oocyst complex, making subsequent detection/confirmation easier. The addition of kaolin to improve oocyst recoveries during IMS was found to be beneficial only in raw and primary effluent concentrates.

The use of *ColorSeed* as an internal control in wastewater samples to determine method performance was introduced when the product became commercially available. The recovery of the *ColorSeed* oocysts was compared to the recovery of seeded live oocysts in raw wastewater since live oocysts were used up initially to assess method performance. Statistical analysis comparing recovery rates of *ColorSeed* to seeded live oocysts showed that *ColorSeed* seemed to provide a conservative estimate of how well the method was able to recover seeded live oocysts providing the Dynal IMS kit is used. Because of the complex and every changing nature of the wastewater matrices, *ColorSeed* can serve as a tool for method performance in the matrix on a given day. It may indicate a negative interference, as noted

during method development trials when no seeded oocysts were recovered when dairy waste was dumped in to the waste stream or relatively high recoveries noted in the primary effluent when waste from chemical toilets were dumped into the wastewater plant (Table 2, trial 2). However, *ColorSeed* recoveries should not be used as a concentration calculation factor to predict the concentrations of indigenous oocysts in these matrices since we cannot predict the ability of the method to recover them. Chemical and physical parameters may play a role in IMS method performance but determining their impact on oocyst recoveries was beyond the scope of this project.

Based on the statistical analyses performed on each wastewater data set it appears the Dynal IMS kit offers improved performance under most test conditions. With the Dynal *Cryptosporidium* IMS kit the differences in recoveries were insignificant as the volume analyzed increased from 250 mL to 1 L. With the Aureon IMS kit, significant differences in oocyst recoveries in raw influents were noted when sample volumes analyzed were increased from 250 to 500 mL. However the differences noted in oocyst recoveries when the sample volume was increased to 1 L was not significant using the Aureon kit. Significant differences in oocyst recoveries in the “cleaner” matrices (secondary and tertiary effluents) were more pronounced when comparing the recoveries with the two IMS kits. In IMS kit comparison trials other researchers have reported differences in oocyst recoveries in environmental matrices (Bukhari et al., 1998; Rochelle et al., 1999) with the Dynal IMS kit outperforming other IMS kits tested. Overall oocyst recoveries as well as the variability of those recoveries improved as the level of particles in the wastewater matrix declined. These results are not unexpected since tertiary effluents are relatively clean with reduced levels of chemical and physical interferences.

When a direct IMS procedure was used to isolate *C. parvum* oocysts in biosolids, with a total solids content of approximately 10%, mean recovery rates were greater than 40%. Unlike raw wastewater, the use of 0.75 g kaolin in wastewater sludge did not improve IMS performance. Increasing the sample size to 10 or 15 g and analyzing as two subsamples using IMS decreased oocyst recoveries further (Table 4),

suggesting that increasing particle concentrations probably impacted IMS performance.

A dual isolation procedure was considered to improve oocyst recoveries in biosolids. This method involved subjecting the biosolids to density gradient flotation followed by subjecting the harvested interface to IMS. Mean oocyst recoveries in the St. Albans sludge dropped to $25.9 \pm 6.1\%$ using this approach. However, in the ALCOSAN dewatered sludge the mean recoveries improved to $35.7 \pm 10.0\%$. In the ALCOSAN lime-stabilized sludge samples, a gelatinous floc was noted after flotation, which may have interfered with oocyst isolation during IMS. These observations suggest that in addition to the concentration of particles, chemical composition of the sludge may also be a factor affecting IMS performance. Massanet-Nicolau (2003) reported on a similar approach in isolating *Giardia* cysts and *Cryptosporidium* oocysts in biosolids. The author observed oocysts recoveries of approximately 5% when digested sludge was subjected to sucrose flotation (sp. gr. 1.18) followed by IMS with spike doses of 10^2 . However, these low recoveries may be a result of using formalin preserved organisms. Bukhari and Smith (1995) demonstrated that sucrose flotation selectively isolates viable oocysts. Since the organisms used by Massanet-Nicolau were formalin-preserved this may account for the low recoveries observed. At spike dose concentrations of $2 \times 10^3 \text{ g}^{-1}$, Kuczynska and Shelton (1999) observed mean oocyst recoveries ranging from <1% to 18.7% in calf feces. In this study, sucrose flotation yielded a mean recovery of $17.9 \pm 2.7\%$.

Other approaches were investigated but results were not reported due to poor oocyst recovery rates achieved in trials conducted. Slow speed centrifugation and coarse filtration were explored as measures to reduce particle levels in biosolids samples prior to IMS. However, these approaches were considered unacceptable, as oocyst recoveries were generally poor (<10%). In addition, the White House draft method (USEPA, 1992) for *Ascaris* ova recovery was modified to evaluate recovery of *Cryptosporidium* oocysts from biosolids. This method was not only cumbersome, but utilized various manipulations, resulting in numerous stages in the procedure for potential loss of oocysts. In fact, oocyst recovery was <10% when this modified procedure was evaluated.

Modifications to existing oocyst recovery protocols were explored to increase sample size for analysis or to improve overall recoveries in biosolids. These included splitting the sample between two IMS tubes and using a panning magnet for matrices with higher solids content, but recoveries did not improve. Using other techniques, such as slow speed centrifugation and coarse filtration also failed to improve oocyst recoveries from biosolids samples. In these trials, the only procedure that has shown acceptable oocyst recoveries relies on using small sample weights (<5 g). However, there may be issues with this procedure when analyzing biosolids with a high percent solids or added chemical stabilizers. These trials, and recovery data reported by other researchers, continue to prove how difficult these matrices are to work with.

In conclusion, the methods reported in this paper demonstrate that acceptable oocyst recoveries can be achieved in wastewater utilizing IMS to isolate the oocysts from the complex matrix. In raw influents and primary effluents the incorporation of kaolin in the IMS procedure can improve recoveries of seeded organisms. In secondary and tertiary effluents, procedures outlined in Method 1622 can be followed with the inclusion of a pre-elution step with 5% NaHMP. In some biosolids, acceptable oocyst recoveries can be achieved by directly subjecting the biosolids to IMS. By including an internal control, *ColorSeed*, with every sample, method performance can be evaluated. If interferences are present that inhibit the performance of the IMS kit, the internal control recoveries will indicate such. In addition, *ColorSeed* oocysts can be easily distinguished from indigenous oocysts by the inclusion of a red dye that is detected during the microscopy phase of the assay. However, *ColorSeed* results should not be used to calculate the concentrations of indigenous oocysts since we cannot determine the ability of the method to recover these environmentally stressed organisms. One important step left in wastewater method development is to determine if recovered indigenous oocysts are infectious, by employing cell culture techniques to ascertain public health significance.

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