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Occurrence of *Cryptosporidium* oocysts in US wastewaters

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ABSTRACT

Wastewater samples collected from ten wastewater facilities across the US were analyzed to determine the occurrence of indigenous *Cryptosporidium* oocysts using methods based on modifications of USEPA Method 1622. Wastewater facilities participating in this study ranged in size from 0.6 to 193 mgd average daily flow. A total of 289 wastewater samples were analyzed over a 15-month period. *Color*Seed is a commercial product containing gamma-irradiated *Cryptosporidium* oocysts that have been permanently stained with a Texas Red dye. *Color*Seed was used as an internal control with each sample to assess method performance. In 500 ml sample volumes, mean *Color*Seed recoveries in raw influents and primary effluents were 26.1 ± 17.7% and 33.0 ± 17.9%, respectively. In 10 liter volumes of secondary effluent, mean *Color*Seed recovery was 25.0 ± 16.6%. Volumes analyzed for tertiary effluent samples ranged from 14.8 l to 131.3 l resulting in a mean *Color*Seed recovery of 48.8 ± 14.5%. Indigenous oocysts were detected in 30% of raw influents, 46% of primary effluents, 58% of secondary effluents and 19% of tertiary effluents analyzed. Indigenous oocyst concentrations ranged from <2 to 86 /liter across all wastewater matrices tested.

Key words | *Color*Seed, *Cryptosporidium*, detection, methods, occurrence, wastewater

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INTRODUCTION

Chemical treatment of drinking water was once considered sufficient for reducing the risk of infection from pathogenic organisms. However, as our knowledge of established and emerging pathogens in water has expanded, so has the need to examine their occurrence, distribution, risk to humans, and control through treatment. The increased need for wastewater reuse has resulted in attention now being focused on wastewater treatment processes and their ability to reduce the numbers of pathogenic organisms to acceptable levels. A public health concern with wastewater is the potential for transmission of infectious agents that may be present in human and animal feces. Depending upon the diseases in the contributing communities, wastewater can contain varying numbers of pathogenic organisms including viruses, bacteria, helminths and protozoa.

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This paper focuses on one pathogen, *Cryptosporidium parvum*, and its occurrence in wastewater. *Cryptosporidium* is a significant concern to water suppliers worldwide, as this protozoan parasite forms highly resistant oocysts that can survive in moist environments for extended periods. The oocysts are highly resistant to environmental pressures and are difficult to remove in water treatment due to their small size (4 to 6 μm). *Cryptosporidium* oocysts are resistant to conventional chlorine based disinfectants, and oocysts that escape the coagulation/filtration process can remain viable. Dozens of outbreaks of cryptosporidiosis have occurred worldwide, with deaths resulting.

Animals and humans are reservoirs for this parasite, and it enters the environment through shedding of fecal material. Dozens of species harbor *Cryptosporidium* oocysts including mammals (cattle, horses, rodents, deer,

dogs, cats), kangaroos, birds, reptiles, and fish, although not all species are infectious to humans. As such, there are many routes for this parasite to enter the environment including natural runoff (non-point sources), runoff from agriculture, effluents from industries such as meat processors, wastewater effluents, and combined sanitary and combined sewer overflows.

Following treatment, wastewater effluents are discharged in receiving waters, and these waters in turn may be used as sources of water for human consumption after treatment, or for agricultural or recreational purposes. Wastewater effluents have the potential to contaminate the aquatic environment with oocysts. The presence of small numbers of viable oocysts in potable waters can pose a significant threat of infection to individuals or communities that consume such water. It is important to be able to evaluate the presence, removal, and inactivation of *Cryptosporidium* spp. oocysts through the wastewater treatment processes. Standard methods for determining the presence of *Cryptosporidium* oocysts in wastewater matrices were developed and tested recently (McCuin & Clancy 2005). These methods were used by the authors to collect and analyze samples from wastewater utilities. This manuscript presents the results of a 15-month survey of *Cryptosporidium* occurrence in ten US wastewater treatment plants.

The objectives of the study were 1) to collect and analyze wastewater samples from ten plants in the US to determine occurrence of *Cryptosporidium* oocysts in various matrices from raw influent to tertiary effluent using methods designed specifically for recovery of oocysts from wastewater matrices, and 2) to assess oocyst removal through the treatment process.

Description of wastewater treatment facilities

Ten plants in six states were included in the survey. Each plant is described briefly.

Alabama

None of the three Alabama (AL) plants uses chemicals, such as polymers, alum, or caustic for enhanced coagulation, phosphorus removal or pH adjustment. AL Plant #1 operates at 16 to 18 million gallons per day (mgd) with a peak design flow of 35 mgd. The wastewater influent is comprised of domestic waste (70%) and industrial waste (30% bakery and chicken/cattle slaughter houses). Influent wastewater is pretreated using screening and grit removal and then enters the primary clarifiers. The primary weir effluent flows to the secondary clarifiers where more settling occurs for particle removal (aeration, 6 hr detention). The final effluent remains in a chlorine contact basin for 30 minutes before being discharged to receiving waters. The chlorine residual in the final effluent is approximately 0.1 mg/l with a maximum residual of 0.37 mg/l. AL Plant #2 operates at 10 to 12 mgd with a peak flow design of 21 mgd. The wastewater influent is comprised of 60% domestic and 40% industrial (surgical implants manufacturing, sheet metal, food industry). Preliminary treatment of the influent includes screen and grit removal as well as pre-aeration. The pretreated wastewater enters the primary clarifiers and the weir effluent is passed through trickling filters. The trickling filter effluent enters the secondary clarifiers. The effluent from the secondary clarifiers remains in a chlorine contact basin for 30 min before being discharged into receiving waters. AL Plant #3 operates at 2 mgd with a peak flow design of 3 mgd. The wastewater influent is comprised of 70% domestic and 30% industrial (food industry). Preliminary treatment of the influent includes screen and grit removal as well as pre-aeration. The pretreated wastewater enters the primary clarifiers and the weir effluent is passed through biofilters, intermediate clarifiers, and then trickling filters. The trickling filter effluent enters the secondary clarifiers. The effluent from the secondary clarifiers remains in a chlorine contact basin for 30 minutes before being discharged.

California

The CA plant receives 95% domestic and 5% light industrial wastewater with an average daily flow of 17 mgd and a peak daily flow of 30 mgd. This fully combined sewer system may reach peak flows of 65 mgd during the wet weather season. During this time 43 mgd receives secondary treatment and the remainder only primary. The primary effluent is blended with the discharge effluent providing the flow is ≤ 65 mgd. If the flow exceeds 65 mgd, decanted effluent from the outfall is discharged with the plant effluent. Ferric chloride is added to

the secondary clarifiers to aid in the coagulation/flocculation process. In the summer hypochlorite is added to the influent stream to aid in odor control.

Colorado

The CO plant currently operates at an average daily flow of 12 mgd with a maximum flow rate of 17 mgd. The average design flow is 23 mgd with a peak hour flow capacity of 35.6 mgd. The wastewater influent is 96% domestic waste with brewery waste comprising the balance of the influent. The treatment process is conventional activated sludge plant. The secondary clarifier effluent is chlorinated and dechlorinated before discharge from the plant.

North Carolina

The NC Plant is a reclamation facility with an average of 8% of the daily flow being diverted for irrigation use. Primary treatment involves the use of a two-stage phase bar screen and grit removal. A phased isolation ditch provides biological nutrient reduction by alternating the wastewater stream between aerobic and slightly anaerobic conditions to convert ammonia to nitrogen gas and lower the levels of biological oxygen demand (BOD) and phosphorus without addition of chemicals. The wastewater stream flows to the clarifiers for reduction of particles. Clarifier effluent is passed through sand filters for additional particle/biological removal, with the effluent treated with UV light (ave. fluence = 40 mWs/cm^2). Additional disinfection is achieved by dosing with hypochlorite to maintain a residual of 2 ppm. Average daily flow through this plant is 7 mgd with a peak flow capacity of 12 mgd. Domestic wastewater comprises 93% of the wastewater with the balance being pharmaceutical and food processor (cookies, crackers) waste.

Pennsylvania

Pennsylvania (PA) Plant #1 is a conventional secondary treatment facility receiving primarily domestic waste. The average daily flow is 186 mgd with a maximum flow of 420 mgd. Phosphorus is added to the wastewater stream prior to aeration to provide nutrients for the microbial population. Sludge received from a drinking water plant contains high levels of ferric chloride that depletes the available phosphorus in the wastewater. The secondary effluent is chlorinated before pumping the discharge to a river. PA Plant #2 is a conventional secondary treatment facility receiving primarily domestic waste. The average daily flow is 93 mgd with a design flow of 292 mgd. Phosphorus is added to the wastewater stream prior to aeration to provide nutrients for the microbial population. Sludge received from a drinking water plant contains high levels of ferric chloride that depletes the available phosphorus in the wastewater. The secondary effluent is chlorinated before discharging into a river. PA Plant #3 receives primarily domestic waste with about 10% being industrial. This plant uses high purity oxygen in the primary clarifiers to maximize the oxidation rate of iron and manganese in the wastewater. The average daily flow is 193 mgd with a peak daily flow of 451 mgd. The secondary effluent is chlorinated and then discharged to a river.

Vermont

The Vermont (VT) advanced aeration plant has an average flow of 0.6 mgd with a peak flow design of 1.25 mgd. Wastewater influent is comprised of domestic waste (90%), and effluent from a microbrewery. The raw influent travels directly to the aeration tanks after grit removal. The aeration tank outfall is treated with alum, which is applied at a dose of 5 mg/l for phosphorus removal and then flows on to the secondary clarifiers. The clarifier effluent (secondary effluent) is passed through a cloth filter (10–20 μm) and passes by two banks of UV lights before exiting the plant.

METHODS

Collection of wastewater samples

Sample instructions, data sampling sheets, sample bottles, filters, *Color*Seed, 0.05% Tween 80 and coolers with ice packs were sent to utility personnel. One utility did not have the equipment or resources to filter their secondary effluent samples and collected grab samples in cubitainers and shipped them on ice to the laboratory. Raw influent and primary effluent samples were collected as 1 liter samples. Samples from all facilities were grab samples with the

exception of those collected at the PA plants, which were 24 h composites. Pall Envirochek™ HV filters (Pall Corp. Ann Arbor, MI, product no. 12099) were used to capture and retain oocysts in secondary and tertiary effluents. The capsule was filled in the laboratory with deionized water (DI) and the contents of one vial of *Color*Seed was transferred to the HV filter through the inlet port using the procedure prescribed by the manufacturer. The capsule filter was then connected to the sampling apparatus. One end of the inlet line was submerged in the secondary or tertiary effluent waste stream and the other end was connected to the inlet of the filter. The inlet of the capsule filter was elevated to avoid draining the contents out of the inlet during sample collection. The filter outlet was connected directly to a centrifugal pump equipped with a flow restrictor and flow totalizer. Ten-liter volumes of secondary effluent were filtered through the capsule filter HV filter at a flow rate of 2 l·min^{-1}.For tertiary effluents sample collection was completed when the flow rate dropped below 0.5 l·min^{-1}.Samples were transported to the laboratory by overnight courier or on blue ice by laboratory personnel (VT samples) and analyzed within 24 hours of receipt.

*Color*Seed™ flow-Sorted spike dose suspensions

*Color*Seed is a commercially available product from Biotechnology Frontiers Pty, Ltd (BTF, PO Box 599, North Ryde BC, NSW 1670, Australia). This product contains flow cytometer-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 have a shelf life of 4 months if stored at 2–8°C. *Color*Seed 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 *Color*Seed 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 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.

Analysis of raw influent and primary effluent

Procedures for analysis of the wastewater samples were previously described (McCuin & Clancy 2005). Briefly, a 500 ml volume of raw influent or primary effluent was transferred to a polypropylene bottle capable of holding 550 ml. One vial of *Color*Seed was added using the transfer protocol prescribed by the manufacturer. In addition, 25 ml of a 20% Tween 80 solution was added to yield a final concentration of 1% and was then thoroughly mixed. The sample was concentrated by centrifugation (1,500 × 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 to 8 ml. *C. parvum* oocysts in the concentrates were isolated using Dynal's anti-Cryptosporidium IMS kit as described below and recovered organisms were enumerated using epifluorescence microscopy.

Analysis of secondary and tertiary effluents using HV filters

A pre-elution step was performed on the secondary and tertiary effluents by adding a 5% (weight per volume) solution of sodium hexametaphosphate (NaHMP, 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 minutes. The solution was allowed to drain through the filter, 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 allowed to drain through the filter membrane, continuing the purge of dissolved or sub-micron material. These preliminary elution steps were followed by the standard 1622 procedure (USEPA 2001). 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 minutes 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 (1,500 × g; 15 min.). The supernatant was aspirated to 5 to 8 ml and quantitatively transferred to a Leighton tube containing IMS buffers. *C. parvum* oocysts in the concentrates were isolated using the Dynal anti-Cryptosporidium IMS kit as described below and recovered organisms were enumerated using epifluorescence microscopy.

IMS procedure

Each wastewater 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 anti-Cryptosporidium IMS kit (Dynal Biotech ASA 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. Kaolin (0.75 g) (Sigma Chemical, Inc., St. Louis, MO catalog no. K7375) was added to raw influent and primary effluent concentrates. No kaolin was added to concentrates of secondary or tertiary effluents. To each tube, 100 μL of the Crypto Dynabeads were added and incubated for 1 hour while rotating on a sample mixer (18 rpm) at room temperature. At the end of the incubation period, the beads were concentrated by placing the LT in an MPC-1 magnet 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 minute and then allowed to stand undisturbed for 3 minutes. At the end of 3 minutes, the tube was gently rocked in the MPC-1 for 30 seconds. 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 placed back in the MPC-1.The tube was gently rocked for 1 minute and again allowed to stand undisturbed for 3 minutes. The tube was gently rocked for 30 seconds and the rinse decanted. For secondary and tertiary effluents, the LT was gently rocked through a 90° arc for 2 minutes and the supernatant decanted. For all samples, the bead-oocyst complex was resuspended in 1 ml of 1X SL™-buffer A and transferred to an individual 1.5 ml Eppendorf tube. Another volume of 1 X SL-A buffer (500 μl) was added to the LT tube to remove residual debris and was then added to its respective 1.5 ml Eppendorf tube. Each tube was placed in the MPC-M magnet with the magnetic strip in place and was rocked 180° for 2 minutes 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 μl 0.1N HCl, vortexed and incubated for 5 minutes 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 μl 1.0 N NaOH. After drying at 42°C on a slide warmer, the sample was methanol-fixed and stained with fluorescein isothiocyanate anti-Cryptosporidium monoclonal antibodies (FITC-mAb) and 4′ 6-diamidino-2-phenylindole (DAPI) solution (0.4 μg• or $\cdot ml^{-1}$ in PBS) as previously described (McCuin and Clancy, 2005). The slides were examined using epifluorescence microscopy.

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 360X. DAPI staining characteristics were observed at 640X 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 *Color*Seed at 640X magnification. Internal morphology of oocysts was observed by using Nomarski DIC microscopy at 640–1600X magnification.

Sample turbidity measurements

For secondary and tertiary effluents filtered in the field, field personnel provided turbidity values. The turbidity of each

sample matrix was measured prior to processing for turbidity measurements performed in the laboratory. A HACH 2100P turbidimeter was used to determine turbidities measured in the laboratory. This turbidimeter is capable of measuring turbidity levels between 0 and 1,000 ntu. If necessary, sub-samples of the original matrix were diluted in DI to enable turbidity measurements. The appropriate dilution factor was used to determine the turbidity of the original sample.

RESULTS

Table 1 presents *Color*Seed recovery data, turbidities and indigenous oocyst concentrations detected in wastewater samples collected at ten wastewater treatment plants in the US from May 2002 through July 2003.

Alabama

*Color*Seed recoveries in raw influents ranged from 0 to 83.8% with the highest mean recoveries of 44.2 ± 18.0% obtained at AL Plant #1. Indigenous oocyst concentrations in raw influents ranged from <2 to 24 oocysts /liter with the highest concentrations at AL Plant #1. *Color*Seed recoveries in primary effluents ranged from 5.1% to 78.8% with the highest mean recovery noted at AL Plant #1 with 48.9 ± 9.9%. The highest levels of indigenous oocysts were detected at AL Plant #2 with 82 oocysts /liter. Filtration of 10 liter sample volumes of secondary effluents was achieved using one Envirochek HV filter with the exception of the one sample collected at AL Plant #3 when two filters were required to collect a total volume of 10 liters. Mean *Color*Seed recovery at AL Plant #1 was 18.6 ± 21.9% with recoveries ranging from 2.0% to 63.0%; 19.5 ± 18.9% with recoveries ranging from 0 to 47.5% at AL Plant #2 and 27.3 ± 12.1% with recoveries ranging from 9.1% to 47.5% at AL Plant #3. Concentrations of indigenous oocysts detected in secondary effluent ranged from <0.1 to 14.5 oocysts/liter.

California

*Color*Seed recoveries in the raw influent were less than 15% with the exception of one sample analyzed in July where the *Color*Seed recovery was 27.8%. Sufficient sample volume of each matrix was collected in one month to examine variability of the matrix. Triplicate subsamples analyzed showed very consistent *Color*Seed recoveries and the number of indigenous oocysts detected was 7.1 ± 0% (n = 3) and <2/liter, respectively (data not shown). The *Color*Seed recoveries in primary effluents analyzed were much improved over the raw influent results with recoveries ranging from 0% to 68.7% and a mean of 37.3 ± 20.2%. A high level of variability was noted in the triplicate subsamples analyzed. *Color*Seed recoveries ranged from 0% to 68.7%, while concentrations of indigenous oocysts detected ranged from <2/liter to 24/liter in the triplicate analyses of the primary effluent samples. Indigenous oocysts were detected in all other primary effluent samples analyzed from this plant. The concentrations detected ranged from 2/liter to 86/liter. The highest level of indigenous oocysts detected in the primary effluents was from a sample collected in December, which is the same month that the highest levels were noted in the secondary effluent at 39/liter (data not shown). Indigenous oocyst concentrations in the secondary effluents ranged from 0.1/liter to 39/liter. The mean *Color*Seed recovery for the secondary effluent samples analyzed was 24.8 ± 19.0% (n = 12) and ranged from 1.0% to 62.6%. Triplicate subsample analyses were more consistent than in primary effluent samples with *Color*Seed recoveries ranging from 16.2% to 28.3% and indigenous oocyst concentrations ranging from 1.7/liter to 1.9/liter. These concentrations of indigenous oocysts at this plant represent the highest concentrations detected for all plants participating in this survey.

Colorado

No indigenous oocysts were detected in the raw influent samples analyzed at the secondary treatment facility in Colorado. The mean *Color*Seed recovery was 11.8 ± 8.7% with recoveries ranging from 3.0% to 25.3%. *Color*Seed recoveries in primary effluent ranged from 1.0% to 51.0% with a mean recovery of 23.0 ± 13.8%. Indigenous oocysts were detected in primary effluent samples analyzed with concentrations ranging from <2/liter to 24/liter. In secondary effluent indigenous oocysts were detected in all samples analyzed with concentrations ranging from

Table 1 | *Color*Seed recoveries and indigenous oocysts detected in wastewater samples collected in ten US wastewater treatment facilities

State	Matrix (# of samples)	Range of turbidities (ntu)	Mean percent *Color*Seed recoveries (Range)	Range of indigenous *C. parvum* oocysts detected (#/L)
AL				
#1	Raw (11)	52.9–155	44.2 ± 18.0 (17.2–83.8)	<2–24
	Primary effluent (11)	41.3–70.1	48.9 ± 9.9 (34.3–65.7)	<2–8
	Secondary effluent (11)	1.0–4.6	18.6 ± 21.9 (2.0–63.0)	<0.1–1.0
#2	Raw (11)	101–700	35.0 ± 18.2 (12.1–73.7)	<2–2
	Primary effluent (11)	55.2–120	44.9 ± 19.6 (8.1–78.8)	<2–82
	Secondary effluent (11)	3.8–17.4	19.5 ± 18.9 (0–47.5)	<0.1–14.5
#3	Raw (11)	79.1–563	23.4 ± 15.9 (0–46.5)	<2–2
	Primary effluent (11)	10.9–49.2	37.0 ± 15.1 (5.1–52.5)	<2–2
	Secondary effluent (11)	2.9–18.9	27.3 ± 12.1 (9.1–47.5)	<0.1–3.6
CA				
	Raw (12)	35.3–269	9.0 ± 7.4 (0–27.8)	<2–24
	Primary effluent (12)	36.3–80.9	37.2 ± 20.2 (0–68.7)	<2–86
	Secondary effluent (12)	1.5–11.5	24.8 ± 19.0 (1.0–62.6)	<0.09–38.9
CO				
	Raw (9)	89.2–261	11.8 ± 8.7 (3.0–25.3)	<2
	Primary effluent (9)	42.7–156	23.0 ± 13.8 (1.0–51.0)	<2–24
	Secondary effluent (9)	2.1–5.9	30.5 ± 18.5 (6.1–52.0)	0.1–0.7
NC				
	Raw (5)	149–529	16.7 ± 11.5 (3.0–29.2)	<2–2
	Primary effluent (5)	130–327	11.5 ± 5.5 (6.1–19.2)	<2
	Secondary effluent (5)	0.8–1.7	43.1 ± 17.6 (25.3–70.0)	<0.09–0.62
	Tertiary treatment (5)	0.8–1.7	44.0 ± 21.1 (19.2–65.7)	<0.04–0.23
PA				
#1	Raw (7)	61.4–152	18.4 ± 10.5 (10.1–40.0)	<2–6
	Primary effluent (7)	33.4–91.3	28.1 ± 9.3 (16.2–45.0)	<2–8
	Secondary effluent (7)	1.7–6.4	26.6 ± 15.3 (7.1–50.6)	<0.1–3.2
#2	Raw (8)	45.4–101	28.7 ± 11.0 (14.1–48.5)	<2–4
	Primary effluent (7)	18.5–178	21.8 ± 12.2 (5.1–39.0)	<2–2
	Secondary effluent (8)	0.7–2.7	32.6 ± 16.3 (8.0–55.6)	<0.1–0.71
#3	Raw (10)	34.3–141	29.5 ± 8.1 (19.2–34.3)	<2–8
	Primary effluent (10)	39.3–83.5	29.1 ± 9.0 (15.2–44.4)	<2–8
	Secondary effluent (9)	0.8–8.7	22.4 ± 13.9 (8.1–43.4)	<0.1–3.7
VT				
	Raw (11)	72.3–296	42.0 ± 14.0 (17.2–72.0)	<2–22
	Secondary effluent (11)	1.6–10.9	30.4 ± 13.7 (2.0–49.5)	<0.08– < 0.1
	Tertiary effluent (11)	0.8–2.6	51.8 ± 7.7 (35.4–62.6)	<0.008– < 0.07

*Color*Seed spike dose concentrations: 99 or 100 oocysts.
Indigenous oocysts are those present in the wastewater sample.

0.1/liter to 0.7/liter. The mean *Color*Seed recovery in secondary effluent was 30.5 ± 18.5% with recoveries ranging from 6.1% to 52.0%.

North Carolina

*Color*Seed recoveries in raw influent samples collected at a tertiary treatment plant in North Carolina ranged from 3.0% to 29.2% with a mean recovery of 16.7 ± 11.5%. Indigenous oocysts were only detected in one raw influent sample at a concentration of 2/liter (data not shown). Mean *Color*Seed recovery in primary effluent was 11.5 ± 5.5% and no indigenous oocysts were detected in these samples. *Color*-Seed recoveries in secondary effluent samples ranged from 25.3% to 70.0% for a mean recovery result of 43.1 ± 17.6%. Indigenous oocysts were detected at concentration ranging from < 0.09/liter to 0.62/liter. Mean *Color*Seed recovery in tertiary effluent was 44.0 ± 21.1% with recoveries ranging from 19.2% to 65.7%. Indigenous oocysts were detected in three of four tertiary samples analyzed with concentrations ranging from < 0.04 to 0.23/liter.

Pennsylvania

Wastewaters from three secondary treatment facilities in Pennsylvania were received from November 2002 through July 2003 although samples were not received from Plant #1 in March. All samples were subsamples from 24 hour composite samples. Triplicate subsample analyses were performed on the raw influent and primary effluent samples and duplicate analyses were performed on the secondary effluent on samples collected at PA Plant #3 during one month. *Color*Seed recoveries in raw influent were lowest at PA Plant #1 with recoveries ranging from 10.1% to 40.0% and a mean recovery of 18.4 ± 10.5%. The mean *Color*Seed recovery in raw influents at PA Plant #2 was 28.7 ± 11.0% with recoveries ranging from 14.1% to 48.5%. At PA Plant #3 *Color*Seed recoveries ranged from 19.2% to 47.5%, yielding the highest mean *Color*Seed recovery of the PA plants at 29.5 ± 8.1%. The analysis of triplicate subsamples of raw influent in May yielded recoveries ranging from 20.2% to 31.3% with a mean recovery of 26.3 ± 5.6% (data not shown). Indigenous oocysts were detected in raw influent samples at all plants with the highest concentrations noted at PA Plant #3 with the concentration ranging from < 2/liter to 8/liter.

At PA Plant #1 the mean *Color*Seed recovery in primary effluents was 28.1 ± 9.3% with indigenous oocysts being detected in 5 of 6 samples (data not shown). Concentrations of indigenous oocysts detected ranged from < 2/liter to 8/liter in the primary effluent at PA Plant #1. At PA Plant #2 the concentration of indigenous oocysts was lowest of the Pennsylvania plants, with only one sample positive for indigenous oocysts. The mean *Color*Seed recovery at PA Plant #2 was 21.6 ± 13.2% and recoveries ranged from 4.0% to 39.0%. At PA Plant #3 the mean *Color*Seed recovery was 29.1 ± 9.1% and recoveries ranged from 15.2% to 44.4%. Indigenous oocysts were detected at concentrations ranging from < 2/liter to 8/liter. Triplicate analyses on the primary effluent yielded *Color*Seed recoveries ranging from 31.3% to 35.4% with a mean recovery of 33.0 ± 2.1% (data not shown). Indigenous oocysts detected in these triplicate analyses were detected at concentrations of < 2/liter, 2/liter and 4/liter.

Results of *Color*Seed recoveries in secondary effluent samples show recoveries ranging from 1.0% (Plant #3) to 55.6% (Plant #2). Mean *Color*Seed recoveries were 26.6 ± 15.3%, 32.6 ± 16.3% and 22.4 ± 13.9% for Plants #1, #2, and #3, respectively. Duplicate analyses on secondary effluent samples collected at PA Plant #3 yielded a mean *Color*Seed recovery of 37.9 ± 7.8% (data not shown) and 10 indigenous oocysts were detected in each 10 liter sample subsample. Concentrations of indigenous oocysts detected at PA Plant #1 ranged from < 0.1/liter to 3.2/liter, < 0.1/liter to 0.3/liter at PA Plant #2 and < 0.1/liter to 3.7/liter at PA Plant #3.

Vermont

No indigenous oocysts were detected in any of the VT samples collected from the advanced aeration plant with the exception of one raw influent sample. *Color*Seed recoveries in the raw influent ranged from 17.2% to 72.0% with a mean *Color*Seed recovery of 42.0 ± 14.0%. Mean *Color*Seed recovery in the secondary effluent was 30.4 ± 13.7% with recoveries ranging from 2.0% to 50.5%. For one secondary effluent sample, the *Color*Seed recovery was 2.0%; however, during sample collection some of the

contents of the capsule were lost. This may account for the poor recovery result noted this month. The mean *Color*Seed recovery without this data point is 33.3 ± 10.5%. Sample volumes of tertiary effluent ranged from 14.8 liters to 131.3 liters. The sampler allowed collection of the tertiary effluent until the filter plugged. A long period of subfreezing weather in January 2003 and February 2003 prevented adequate particle removal in the wastewater treatment process preventing the collection of higher volumes of tertiary wastewater. Mean *Color*Seed recovery was 51.8 ± 7.7% with oocyst recoveries ranging from 35.4% to 62.2%.

Table 2 summarizes the occurrence survey results for each of the matrices. Turbidity values for raw influent samples ranged from 34.3 to 700 ntu. *Color*Seed recoveries ranged from 0 to 83.8% with a mean recovery of 26.1 ± 17.7%. Indigenous oocysts were detected in 29.5% of raw influent sample analyzed, at concentrations ranging from <2/liter to 24/liter. In primary effluents *Color*Seed recoveries ranged from 0% to 78.8% with a mean *Color*Seed recovery of 33.0 ± 17.9%. The turbidity of the primary samples analyzed ranged from 4.08 to 327 ntu. Indigenous oocyst concentrations ranged from <2/liter to 86/liter with 45.8% of oocysts detected in 45.8% of these samples. In secondary effluents *Color*Seed recoveries ranged from 0% to 62.6%. A mean *Color*Seed recovery of 25.0 ± 16.6% was realized for this matrix. Indigenous oocyst concentrations were found to be <0.1/liter to 40.8/liter in 58.5% of secondary effluents analyzed. In a majority of these samples, 10 liters of secondary effluent could be collected on a single Envirochek HV filter. Of 94 samples collected, only six had sample volumes less than 10 liters. Tertiary effluent samples had the highest mean *Color*Seed recovery of 48.8 ± 14.5% with recoveries ranging from 13.1 to 65.7%. Indigenous oocyst concentrations ranged from <0.008/liter to 0.226/liter and were detected in 18.8% of tertiary effluent samples analyzed. Tertiary effluent samples were collected at two plants (VT and NC), but indigenous oocysts were detected only in the tertiary effluent from the plant in NC.

After compiling the occurrence data, the second objective of this survey was to assess removal of the indigenous oocyst concentrations through the wastewater treatment process. Figures 1–9 present these data. Data from the Vermont plant are not presented in a figure because oocysts were detected only once at this plant in the raw influent, and never in the secondary or tertiary effluents. Figures 1–3 represent oocyst concentrations detected at the three Alabama wastewater treatment plants. Samples were collected from May 2002 through April 2003 with no samples collected in February 2003. Log removals are generally less than 1.0, indicating poor overall removal through the treatment process. In some cases increases in oocyst concentrations from the raw influent to the final influent were noted. At the California wastewater facility (Figure 4) an increase in oocyst concentrations from the raw influent to the final effluent was observed in five of nine sampling events. However, when comparing the log removal from the primary effluent to the final effluent an increase was noted only twice. Oocyst concentrations from samples collected March 2003 showed an increase when the log removal was calculated using the raw influent and final effluent. However, when the primary effluent was used to determine oocyst removal, there was a 1.5 log reduction in oocyst concentrations. At the

Table 2 | Summary of *Color*Seed recoveries, turbidities, and number of samples with indigenous *Cryptosporidium* oocysts in wastewater samples collected from May 2002 through July 2003

Matrix (n = no. samples analyzed)	Volume analyzed (L)	Range of turbidities (ntu)	Mean percent *Color*Seed recoveries (Range)	Concentration of Indigenous oocysts (#/L)	Percent of samples positive for indigenous oocysts
Raw influent (n = 95)	0.5	34.3–700	26.1 ± 17.7 (0–83.8)	<2–24	29.5
Primary effluent (n = 84)	0.5	4.1–327	33.0 ± 17.9 (0–78.8)	<2–86	45.8
Secondary effluent (n = 94)	4.2–22.7	0.8–19.3	25.0 ± 16.6 (0–62.6)	<0.1–40.8	58.5
Tertiary effluent (n = 16)	22.7–131.3	0.8–2.6	48.8 ± 14.5 (13.1–65.7)	<0.008–0.226	18.8

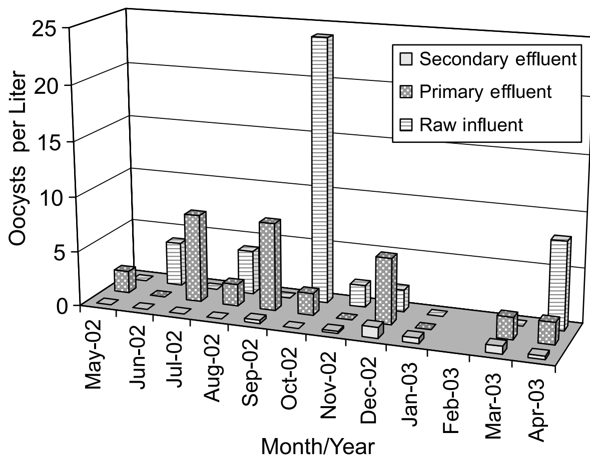

Figure 1 | Indigenous oocyst concentrations at Alabama plant #1 from May 2002 through April 2003.

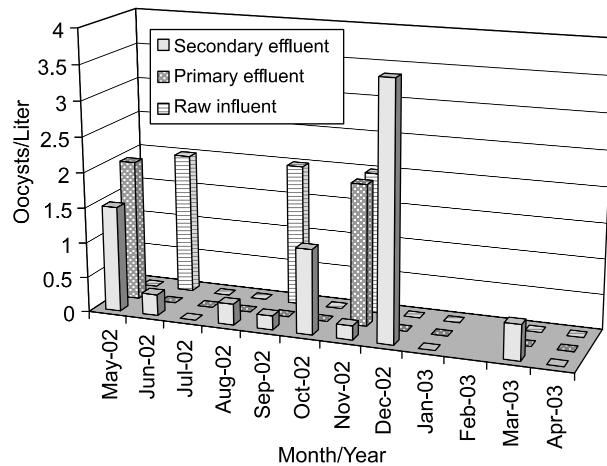

Figure 3 | Indigenous oocyst concentrations at Alabama plant #3 from May 2002 through April 2003.

Colorado facility (Figure 5) log removals were poor when using the raw influent data to calculate removals; however, when using the primary effluent data oocyst removals improved with three datasets to at least 1.0 log and in one case to 2.1 log. At the North Carolina facility (Figure 6) oocyst log removals were poor with an increase in oocyst concentrations from the secondary to tertiary effluent noted once. Data from the Pennsylvania plants (Figures 7, 8 and 9) showed minimal removals.

DISCUSSION

A variety of methods for concentration and isolation of the oocysts have been used to determine the levels of *C. parvum* oocysts in wastewater. These methods have included modifications of the US Information Collection Rule (ICR) or UK Standing Committee of Analysts (SCA) methods (Enriquez *et al.* 1995; Medema *et al.* 1998; Jolis *et al.* 1999; Carraro *et al.* 2000), calcium carbonate flocculation followed by cold sucrose flotation (Payment *et al.* 2001), and coarse filtration of small volumes (50–2,000 ml) through muslin followed by centrifugation and purification using various density gradients and purification solutions (Bukhari *et al.* 1997; Robertson *et al.* 2000). Jolis *et al.* (1999) detected no oocysts in reclaimed water using the ICR method. While recovery efficiencies of the ICR method were not assessed by the Jolis *et al.* study

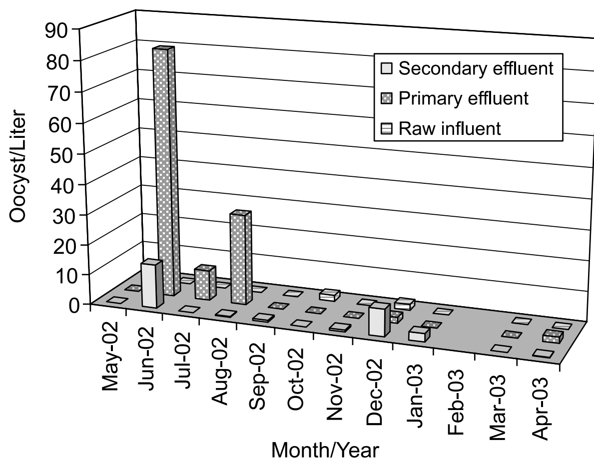

Figure 2 | Indigenous oocyst concentrations at Alabama plant #2 from May 2002 through April 2003.

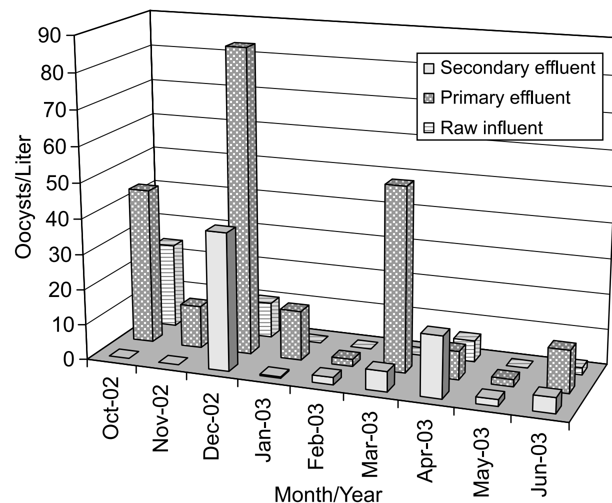

Figure 4 | Indigenous oocyst concentrations at the California plant from October 2002 though June 2003.

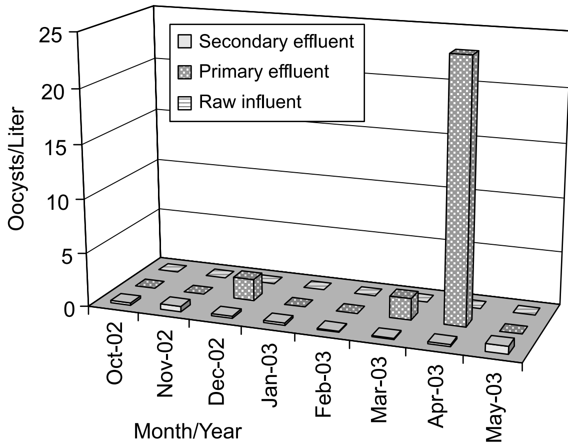

Figure 5 | Indigenous oocyst concentrations at the Colorado plant from October 2002 through June 2003.

(1999), Clancy *et al.* (1994) reported poor recoveries of both *Cryptosporidium* oocysts and *Giardia* cysts, generally less than 10%, when using this method in source waters. Carraro *et al.* (2000) reported detecting *Cryptosporidium* oocysts at concentrations of 4.5 ± 0.8/liter in raw influents (n = 3) and 0.21 ± 0.06/liter in effluents (n = 11) when using the ICR method, but again method recovery was not assessed. Enriquez *et al.* (1995) assessed method recoveries using a modification of the ICR method in DI and reclaimed water. These modifications included increasing the sample collection flow rate to 15 to 19 liters per minute (from 4

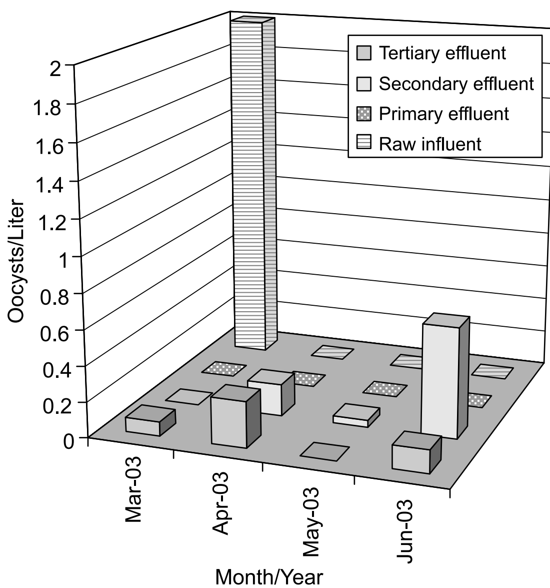

Figure 6 | Indigenous oocyst concentrations at the North Carolina plant from March 2003 through June 2003.

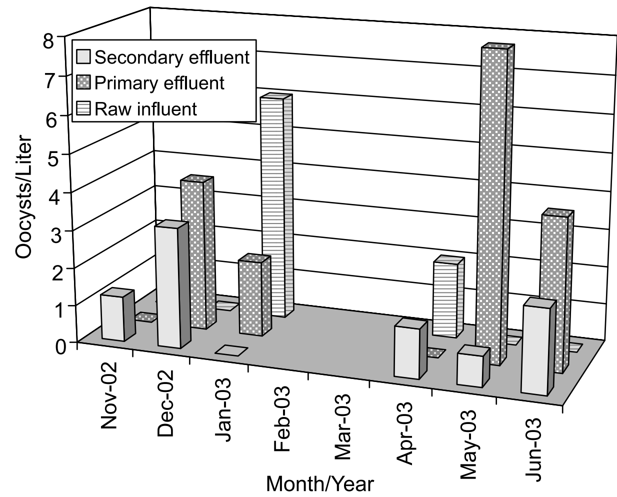

Figure 7 | Indigenous oocyst concentrations at Pennsylvania plant #1 from November 2002 through June 2003.

liters per min.), splitting the concentrated pellet in half, and preserving one half with potassium dichromate for detection of *Cryptosporidium* oocysts. This subsample was subjected to flotation with sucrose (sp gr 1.24) and the interface harvested and applied to cellulose-acetate membrane filters. The mean recovery rate for *Cryptosporidium* in tap water was 9.4 ± 1.4% and 8.7 ± 0.5% in reclaimed water.

Robertson *et al.* (2000) conducted a study of *Cryptosporidium* oocyst and *Giardia* cyst recovery methods using various purification techniques in wastewater in Scotland. The mean recovery rate for *C. parvum* oocysts in wastewater influents at low doses (approximately 120

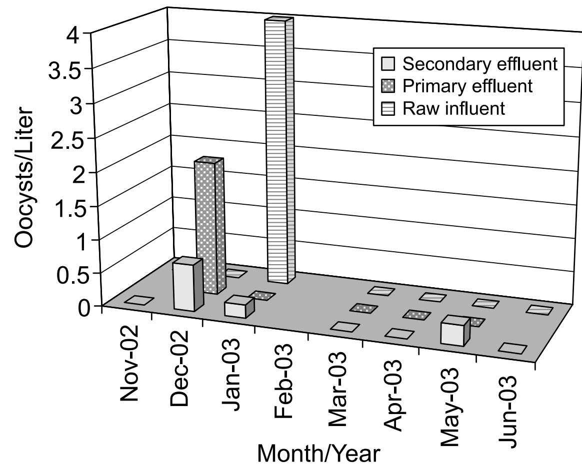

Figure 8 | Indigenous Oocyst Concentrations at Pennsylvania Plant #2 from November 2002 through June 2003.

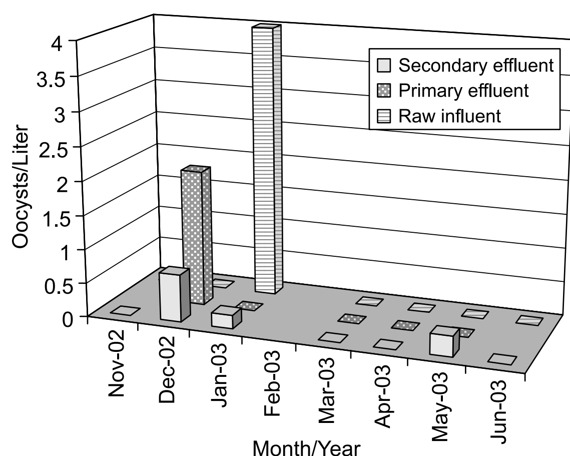

Figure 9 | Indigenous Oocyst Concentrations at Pennsylvania Plant #3 from November 2002 through June 2003.

oocysts/liter) was reported highest (81 ± 21% [n = 8]) when small volumes were subjected to centrifugation followed by direct staining of the pellet material. The mean recovery rates fell to less than 35% when the pellet was subjected to additional manipulations to isolate the oocysts from the contaminating debris. Similar reductions in mean oocyst recoveries were noted when these isolation/ purification techniques were used to recover oocysts from wastewater effluents. The mean oocyst recovery rate fell from 89 ± 10% (n = 8) using direct examination of the concentrate to less than 45% using the three isolation techniques evaluated. After recoveries were assessed, the researchers evaluated the oocyst concentrations at six wastewater treatment facilities in Scotland over a three year period using each concentration/isolation technique. Reported levels of indigenous oocysts at five of six plants ranged from <20/liter to 400/liter in wastewater influents when samples were concentrated and examined without further processing. These levels dropped to <20/liter to 194/liter when further manipulations were conducted. The concentration of oocysts at the sixth plant ranged from 10 to 6,000/liter when subjected to various isolation/purification technique post concentration; however, oocyst levels in the centrifuged pellet were not determined at this plant suggesting that the level of solids present in the pellet was too high to examine directly. In effluent samples Robertson *et al.* (2000) reported oocysts levels of <20/liter to 1,000/ liter when samples concentrates were examined directly. These levels dropped to <20/liter to 205/liter when sample concentrates were subjected to purification/isolation techniques.

Bukhari *et al.* (1997) reported *C. parvum* oocyst concentrations in wastewater influents and effluents collected at seven treatment facilities in the UK. Oocysts were detected in raw influents at concentrations ranging from 10/liter to 170/liter when samples were subjected to coarse filtration, centrifugation, sucrose flotation and further concentration using water and diethyl ether. Oocysts were detected in 27.2% of the raw influents analyzed (n = 81). Wastewater effluents were not subjected to flotation or other purification techniques and were examined directly after concentration by centrifugation. Oocyst concentrations in effluents were reported to be 10/liter to 60/liter with 25.5% of samples analyzed positive for *C. parvum* oocysts (n = 94).

Other occurrence surveys have been conducted to monitor for the presence of *Cryptosporidium* spp. oocysts in raw wastewater and data indicate a range between 4.1 and 13,700 oocysts per liter for the US and 2.5 to 800 oocysts per liter for the UK. The numbers of oocysts recovered from samples, taken from either a single wastewater treatment plant or from different wastewater treatment plants, demonstrate high variability. In raw wastewater, variations in oocyst concentrations are due to numerous factors, including the levels of infection within the contributing community, the severity of disease among the infected individuals, and the size of the community. In a study by Madore *et al.* (1987), it was noted that agricultural discharges contributed the highest numbers of oocysts into raw wastewater (up to 13,700 oocysts per liter). In another UK study, slaughterhouse effluents were determined to be capable of releasing up to 149,100 oocysts per liter (Parker 1993). Because effluents from either cattle markets or abattoirs have the potential to release large numbers of oocysts into raw wastewater, the numbers of oocysts present in raw wastewater are also likely to be influenced by contributions from such sources.

Variations in reported oocyst concentrations may also be due to the methodologies used to isolate and detect them. In most occurrence surveys the concentrates were subjected to isolation and/or purification techniques that are not specific to *Cryptosporidium*. Density gradient flotation procedures can isolate other biological particles

that mask the target organisms during the detection phase of the assay or cross-react with the monoclonal antibodies used to tag *Cryptosporidium* oocysts. Significant numbers of oocysts have been shown to be lost in density gradient centrifugation (Fricker 1995). In addition, it has been reported that some isolation procedures selectively isolate viable oocysts (Bukhari & Smith 1995). McCuin *et al.* (2001) reported that the IMS procedure does not differentiate viable from nonviable oocysts and can recover both at similar rates. In addition, IMS specifically isolates *Cryptosporidium* from interfering debris thereby reducing the number of cross reactors present, and with the inclusion of the DAPI stain that targets the sporozoite nucleic DNA, detection of oocysts in environmental matrices is improved.

Another reason for the variable oocyst recoveries may be due to the inconsistency in the wastewater matrices, especially raw influents and primary effluents, due to high particle levels, fecal fats, oil and grease, and unknown chemical interferences that create challenges for recovering *Cryptosporidium*. The incorporation of the *Color*Seed internal control in this survey was an important tool to evaluate the method performance with each sample. The internal control provided evidence of matrix interference on a day when no seeded oocysts were recovered in a secondary effluent sample. It was later determined that dairy waste from a local dairy cooperative had been introduced into the waste stream. In addition, chemicals present in wastewater, such as iron, may inhibit the performance of the IMS kit (Yakub & Stadterman-Knauer 2000). However, optimizing IMS performance in the presence of these interferences was beyond the scope of this project. In addition, the ability of the IMS kit to recover the *Color*Seed oocysts may not be indicative of the kit's ability to recover environmentally stressed oocysts. Environmentally stressed oocysts may have damaged surface epitopes making their capture by the IMS difficult, or the oocysts may bind with the biological particles rendering the epitopes inaccessible. Some of these interferences appeared to be overcome by the addition of detergent and kaolin to the raw influent and primary effluent samples. Therefore, *Color*Seed recovery data cannot be used to apply a multiplication factor to the number of indigenous oocysts present in a sample for the reasons listed above.

An objective of this survey was to assess indigenous oocyst concentrations through the wastewater treatment process to determine removal. In some cases minimal removal was noted, and in other instances, oocyst concentrations appeared to increase through the treatment process. Since oocysts cannot multiply outside of an animal host, these apparent increases are the result of sampling and method error. It is easier to recover and identify oocysts from cleaner water matrices as evidenced by the increase in percent recoveries from raw influent to tertiary effluent. The cleaner matrices also permit analysis of larger sample volumes. A large number of samples were analyzed in this study, but the sample volumes compared to daily total flow in a plant are miniscule. LeChevallier *et al.* (2002) demonstrated that to develop a reliable protozoan occurrence database, a large number of samples (~100) need to be collected at each sample location to characterize variations and extremes.

A survey conducted in Arizona compared the numbers of *Cryptosporidium* spp. oocysts detected in raw and treated wastewater in a plant that utilized activated sludge, and found oocyst removal efficiencies approached 79% or 0.7 log (Madore *et al.* 1987). It was also noted that plants utilizing sand filtration and activated sludge released significantly lower numbers of oocysts in their final effluents (i.e. 10 oocysts per liter) when compared with plants utilizing activated sludge treatment only (i.e. 1,300 oocysts per liter).

In the UK, Parker (1993) collected six large volume samples (between 100 and 500 liters) of wastewater influent from a municipal treatment works and found oocysts in all six samples at concentrations between 2.5 and 75 oocysts per liter. Samples of treated effluents from 10 wastewater works also indicated the presence of oocysts in 35.1% (25/70) of the samples, with concentrations ranging between 0.024 and 26.5 oocysts per liter. In Parker's study, examination of animal slaughterhouse and poultry slaughterhouse effluents indicated that these sources contributed large numbers of oocysts into wastewater influents (between 92.5 and 149,100 oocysts per liter). For one wastewater treatment plant, comparison of mean and median oocyst concentrations in influent samples (oocyst concentration range from 2.5 to 75 oocysts per liter) and treated effluent samples (0.42 to 26.5 oocysts per liter)

indicated oocyst removal efficiencies of 79.6% and 97.7% respectively.

A Canadian study determined that samples from the five largest population centers in the Yukon contained 26 to 3,022 *Giardia* cysts per liter and 0 to 74 *Cryptosporidium* oocysts per liter, and following wastewater treatment, the occurrence of *Cryptosporidium* oocysts exceeded *Giardia* cysts (Roach *et al.* 1993). These differences in occurrence of *Giardia* and *Cryptosporidium* in raw and treated wastewater may have been due to higher removal efficiencies of the larger sized *Giardia* cysts. It is also possible that the treated wastewater samples contained lower levels of interfering debris, which made identification of the smaller sized *Cryptosporidium* oocysts relatively easier.

To assess the removal efficiency in wastewater treatment works, daily effluent and influent samples from six wastewater treatment works in central Scotland were collected for three years, with the aid of 24 hour samplers (Robertson *et al.* 2000). In order to ensure that effluent samples corresponded with the influent samples, the former were collected after a time delay, which corresponded to the holding time of the treatment works. Data from these investigations indicated that low numbers of *Cryptosporidium* spp. oocysts were detected in both the influent (<10/liter–160/liter) and effluent samples (<5/liter–85/liter), and removal efficiencies were difficult to assess. However, in samples where sufficient numbers of oocysts were recovered for calculation of removal efficiency, wide variations were noted within each treatment works.

A model of an activated sludge treatment plant (flow rate of 17 ml/min. and retention time of 6 hours) was utilized to demonstrate oocyst removal efficiencies of 83.4% and 90.7% following primary and secondary sedimentation respectively (Stadterman *et al.* 1994). In order to evaluate *Cryptosporidium* spp. oocyst removal efficiencies during various wastewater treatment processes, raw wastewater, activated sludge (retention time 2.8 hours), trickling filter (retention time 3.5 hours) and biodisc (retention time 4.8 hours) effluents were seeded with oocysts and settled for 2 hours or for their respective retention times. When compared to other sedimentation processes that were evaluated, activated sludge yielded the highest oocyst removal efficiency, 92% or 1.1 $\log_{10}$ (Stadterman *et al.* 1994).

In this survey the low numbers of oocysts detected in the influent and effluent samples hindered our attempts to make valid statements about removal efficiencies for each of the treatment plants. However, levels of removals determined in this study are similar to those reported by other researchers. In previous studies, methodologies for the isolation of *Cryptosporidium* oocysts in wastewater have relied on nonselective isolation procedures such as density gradient flotation, which can recover algae and other biological particles. These particles may cross react with the monoclonal antibodies or occlude oocysts, preventing proper identification. In addition, identification of oocysts relied primarily on fluorescence pattern, size, and shape. Prior to the late 1990s, very few researchers included the use of DAPI staining or Nomarski DIC optics for visualization of internal structures. Using the older methods (ICR, SCA), the inability to rule out objects similar to *Cryptosporidium* oocysts based on size, shape, and fluorescence patterns alone, likely led to overestimations of the true numbers in samples.

Another problem with the early methods was the analysis of subsamples, where counts in small analyzed volumes were extrapolated to the total volume sampled. Analysis of sub-samples with extrapolation to much larger sampled total volumes (e.g., 100 liters) has been shown to lead to large errors in determination of true pathogen density (Parkhurst & Stein 1998; Atherholt & Korn 1999). This aspect of the early analytical methodology resulted in data that fell generally into two broad categories – either non-detects or very high reported levels based on low analyzed sample volume. For example, with the ICR method, although a 100 liter sample was collected and processed, it was common that a portion equivalent to ~2.5 liter was actually examined microscopically due to method limitations. If one oocyst was observed, then the reported value was 40 oocysts per 100 liter, assuming incorrectly that oocysts are evenly distributed in a sample. The non-detect data are equally absurd. Using the same example, if no oocysts were detected in the 2.5 liters equivalent volume, the count was reported as <40 oocysts per 100 liters. This means the count could be 39 or 0 or any number in between. These methods were so poor that actual count data as well as non-detect data are unreliable (Allen *et al.* 2000). A combination of these

method errors has contributed to the lack of data reliability in the earlier work.

In this study we had the opportunity to utilize IMS to recover oocysts from the wastewater matrices. The IMS technique offers selective isolation resulting in reduced levels of interfering debris. Incorporating an additional wash of the captured bead-oocyst complex prior to disassociation also reduces these interferences. While some non-target organisms may be captured with IMS, the use of the additional fluorogenic stain, DAPI, aids in the identification of recovered oocysts. Incorporating *Color*-Seed in the analysis of every sample offers additional quality assurance/quality control, adding validity to the performance of the method in a given sample. In addition, poor *Color*Seed recoveries may indicate the presence of matrix interferences, which may impact the performance of the IMS protocol to recover indigenous oocysts. In all cases, the volume analyzed was the volume used when reporting oocysts recovered. In the figures, the raw and primary effluent data were doubled (presented as per liter rather than per 500 ml) and the secondary and tertiary effluents were standardized and reported per liter, so that direct comparisons could be made using a standard volume.

CONCLUSIONS

This occurrence survey utilized the most current tools available to recover *C. parvum* oocysts from wastewater. These methods present us with an opportunity to provide QA/QC data on every sample analyzed while offering the most selective oocyst isolation procedure available. These data show that oocysts are present in wastewater matrices at low levels and more importantly are present in effluents being discharged from the plant. The questions that remains to be answered: are these oocysts viable and more importantly, able to cause infection. To determine if oocysts found in wastewater samples present a public health risk, it is necessary to know if they are infectious. The assay used in this survey to detect oocysts is a microscopic assay where both live and dead oocysts are detected, and appear similar morphologically in most instances. There is no way to know if the oocysts observed present a potential public health threat; the current methodology is limited in this aspect and in order to make a risk determination, additional testing to determine infectivity of recovered oocysts is needed. However, the methods to make these infectivity determinations are primarily research tools, with much work needed to develop a reliable, validated method.

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