OCCURRENCE OF *CRYPTOSPORIDIUM* SP. OOCYSTS AND ***GIARDIA*** SP. CYSTS IN SEWAGE EFFLUENTS AND SLUDGES FROM SEWAGE TREATMENT PLANTS IN ENGLAND

Contractor: Scottish Parasite Diagnostic Laboratory

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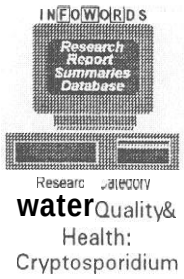

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FR/DW 0002

APRIL 1996

PREPARED FOR
THE DEPARTMENT OF
THE ENVIRONMENT - DRINKING
WATER INSPECTORATE

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FINAL REPORT

OCCURRENCE OF *CRYPTOSPORIDIUM* SP. OOCYSTS AND *GIARDIA* SP. CYSTS IN SEWAGE EFFLUENTS AND SLUDGES FROM SEWAGE TREATMENT PLANTS IN ENGLAND.

EXECUTIVE SUMMARY.

Optimised sample collection and concentration methods were utilised to determine the occurrence of *Cryptosporidium* sp. oocysts and *Giardia* sp. cysts in sewage influent and effluent samples from seven treatment works in England. Five sites where sewage sludge was disposed of onto land were also sampled and analysed for the presence of *Cryptosporidium* sp. oocysts and *Giardia* sp. cysts.

Small numbers of *Cryptosporidium* sp. oocysts were detected in both sewage influent and effluent samples. *Giardia* sp. cysts were detected more frequently and at higher concentrations both in sewage influent and effluents samples. Between 15 and 47% of effluents contained *Cryptosporidium* oocvsts, whereas between 15 and 92% of effluents contained *Giardia* cysts. Our limited data on oocyst viability indicate that viable *Cryptosporidium* sp. oocysts are discharged in sewage effluents.

Sewage sludge samples from one site only contained a mean of 6,700 *Cryptosporidium* oocysts L^{-1}, whereas *Giardia* cyst concentrations between $1x10^4$ and $2.5x10^5$ L^{-1} were detected in sludge samples from all five sites.

Both sewage effluents and sludges can contain *Cryptosporidium* oocysts and *Giardia* cysts. In this study. the highest numbers of oocysts were detected in sewage treatment works which received contributions from either mainly rural, or mainly urban/rural with trade effluents.

The discharge of sewage effluents into a water course which may be used for abstraction for potable water can contaminate that water course with viable oocysts In addition. the application of sewage sludge to land can be responsible for contaminating water courses with *Cryptosporidium* oocysts and *Giardia* cysts following run-off or leaching.

OCCURRENCE OF *CRYPTOSPORIDIUM* SP. OOCYSTS AND *GIARDIA* SP. CYSTS IN SEWAGE EFFLUENTS AND SLUDGES FROM SEWAGE TREATMENT PLANTS IN ENGLAND.

SUMMARY.

Optimised Scottish Parasite Diagnostic Laboratory (SPDL) methods for the recovery of *Cryptosporidium* sp. oocysts and *Giardia* sp. cysts from sewage influent, effluent and sludge samples, which have higher recovery efficiencies than the methods identified in the "Blue book", have been used in this survey of occurrence of oocysts and cysts in sewage intluents. effluents and sludges from seven sewage treatment works in England.

The seven sewage treatment works chosen for the study served communities which had defined contributors of oocysts and cysts. These included "mainly industrial (inorganic), purely residential, urban, mainly industrial (orgamc), with significant flow from an abattoir, with significant flow from a livestock market. rural and possible farm trade effluent and urban/rural and trade effluents".

Statistical analyses of over 150 samples indicated that no significant difference existed between detectton by fluorescence microscopy or detection by flow cytometry followed by fluorescence microscopy for detecting either *Cryptosporidium* sp. oocysts or *Giardia* sp. cysts.

Of a total of 8 1 sewage intluent samples, 27.2% (n = 22) contained between IO - 170 *Cryptosporidium* sp. oocysts L^{-1}. The small numbers of oocysts usually detected in positive samples reduced the amount of information on oocyst viability. However, the sparse data accrued indicate that viable oocysts can be released in sewage effluent discharges. Examination of these sewage intluent samples for the presence of *Giardia* sp. cysts indicated that 77 8% (n = 63) of samples contained between 10 - 33600 *Giardia* cysts L^{-1}. Of a total of94 sewage effluent samples. 25.5% (n = 24) contained between 10 - 60 *Cryptosporidium* sp. oocysts L^{-1} Examinatron of these samples for the presence of *Giardia* sp. cysts indicated that 57 4% (n = 54) of samples contained between 10 - 720 *Giardia* cysts L^{-1}

Removal efficiencies rangmg from 46 4 to 93% were calculated from three treatment works where the numbers of *Cryptosporidium* sp. oocysts were higher in the influents than their respectrve effluents. Removal efficiencies ranging from 26 to 93.8% were calculated for the seven treatment works where the numbers of *Giardia* sp. cysts were higher in the intluents than their respective effluents.

Two treatment works. which received mainly rural and mainly rural/farm trade effluents respectively, contained significantly higher numbers of *Cryptosporidium* sp. oocysts in their intluent samples than the other five studied and it is possible that the oocysts detected in these two sewage treatment works were derived from animal sources. No significant differences could be identified in the numbers of either oocysts or cysts discharged in effluents from any of the seven treatment works studied. Furthermore, the occurrence of *Giardia* cysts in sewage influents could not be associated with any particular contributing source.

Oocysts and cysts were detected in sewage sludge. Oocysts were detected *on* two occasions from one site only, whereas cysts were detected in sludge samples from all five sites on at least one occasion. Thus the potential for introducing oocysts and cysts indirectly into water courses, following the disposal of sewage sludge unto land exists.

INTRODUCTION.

Cryptosporidium parvum is a protozoan parasite capable of infecting both susceptible human beings and domesticated mammals. The life cycle is completed in an individual host, and the auto-infective stages of the life cycle ensure that susceptible individuals release large numbers (up to IO'") of the transmissive stage (the oocyst) in their faeces. The broad host range of *C. parvum*, together with the high output of oocysts ensures a high level of contamination in the environment and both infected human beings and livestock can contribute to the numbers of waterborne oocysts both through sewage effluent discharges, including those containing contributions from abbatoirs. and the disposal of sewage sludge to land.

In the first documented waterborne outbreak of human cryptosporidiosis in the USA (D'Antonio *et* al., 1985), tracer dye investigations implicated sewage as the source of oocyst contamination. The report of the UK group of experts *on Cryptosporidium* in water supplies (Anon, 1990a) Identified both sewage effluents and sewage sludges as sources of oocysts which could contaminate water-courses.

Few studies have examined the occurrence of *Cryptosporidium sp.* oocysts in sewage. Their occurrence is variable. and published data indicate that when oocysts are present in sewage their concentrations can range from 4.1 to 13700 oocysts L^{-1} in studies conducted in the USA (Madore *et al.*, 1987; Rose. 1988) and from 2.5 to 800 oocysts L^{-1} in studies conducted in the UK (Parker *et al.*, 1993; Carrington and Gray, 1993, Dawson *et* al., 1994, Robertson *et* al., in preparation). More occurrence data are available for *Giardia* sp. cysts. Cyst concentrations ranging from 0.075 to 14000 cysts L^{-1} have been documented in studies conducted in the USA (Rose *et* al.. 1988; Sykora *et al.*, 1991; Jakubowski *et al.*, 1991; Enriquez *et al.*, 1995), whereas cyst concentrations ranging from 0.095 to 43907 cysts L^{-1} have been documented in studies conducted in the UK (Parker, 1993; Dawson *et al.*, 1994; Robertson *et al.*, 1995).

Limited information exists regarding the effectiveness of sewage treatment processes for removing and/or inactivating *Cryptosporidium* sp. oocysts. Furthermore. in earlier studies. sewage samples were collected using both large scale filtration and grab samples and because of inconsistencies in both the sample collection and concentration procedures, comparison of results. from these studies, is made difficult. Over the last three years, methods for the isolation of *Cryptosporidium* sp. oocysts and *Giardia* sp. cysts from sewage samples have been developed and optimised at the Scottish Parasne Diagnostic Laboratory (SPDL). These methods, which have higher recovery efficiencies than the methods identified in the "Blue book" (Anon, 1990b), have been used in surveys of occurrence of oocysts and cysts in sewage influents and effluents from six treatment works in the west of Scotland.

The objectives of this project were to monitor the occurrence, removal and inactivation of *Cryptosporidium* sp. oocysts and *Giardia* sp. cysts from sewage treatment works located in England and to determine whether sewage treatment works receiving significant contributions from various identified sources. which could contain oocysts and/or cysts, were more likely to discharge oocysts and/or cysts in their effluents. The sample collection and sample concentration techniques developed and optimised for the Scottish study were utilised in this study in order that the data accrued from this study could be compared with the data accrued from the Scottish study. The

identification of contributors of oocysts into sewage influent was considered to be important, therefore each sewage treatment works selected for investigation had a major contributing source. These included "mainly industrial (inorganic), purely residential, urban. mainly industrial (organic). with significant flow from an abattoir, with significant flow from a livestock market, rural and possible farm trade effluent and urban/rural and trade effluents" (Table I). Initially it was proposed that each sewage treatment works would be sampled at fortnightly Intervals for a period of six months however, due to unforeseen circumstances, our sub-contractors were unable to maintain this sampling schedule. Following correspondence with the nominated person at the Drinking Water Inspectorate, it was agreed that two-thirds of the data collected and reported in this final report could be accrued as a result of intensive sampling of influents, effluents and sludges from the seven English sewage treatment works over a period of l-2 months.

Table 1: Main contributing sources and treatment processes employed in the sewage treatment works under investigation.

Sewage treatment works number.	Main contributing source to sewage treatment works.	Secondary treatment	Tertiary treatment
1.	Mainly industrial (inorganic).	Activated sludge.	N/A
2	Purely residential, urban.	Biological filtration (percolating filters).	Sand filtration.
3	Mainly industrial (organic).	Biological filtration (percolating filters).	Lagoons.
4	Significant flow from an abattoir.	Combination of surface aeration and surface filtration.	N/A
5	Significant flow from a livestock market.	Activated sludge.	Rapid sand filtration.
6	Rural and possible farm trade effluent.	Biological filtration (percolating filters).	N/A
7.	Urban/rural and trade effluents.	Activated sludge (diffused air).	Microstrainers.

N/A = No Tertiary Treatment employed.

Sewage sludge site number	Sewage sludge treatment process and storage time prior to land application
SS1	Raw sludge direct to land (no storage)
ss2	Raw pressed cake (no storage)
ss3	Sludge digested and stored for a minimum of 14 days
ss4	Sludge digested and stored for a minimum of 14 days
SS5	Sludge digested and stored for 6 months

MATERIALS AND METHODS.

SAMPLING AND ANALYSES STRATEGIES ADOPTED IN THIS STUDY.

All sampling was undertaken by our sub-contractors following written protocols identified by the SPDL. All methods used for the isolation and concentration were those developed for the Scottish study (see below), with the exception of the protocol used for the isolation and enumeration of organisms from sewage sludge which was developed at the SPDL. Our sub-contractor undertook all the analyses, with the exception of isolation by flow cytometry and enumeration by fluorescence microscopy, according to defined SPDL protocols. In this manner, the results of samples enumerated by either fluorescence microscopy alone (performed at the SPDL) could be compared statistically with the results of samples enumerated following flow cytometry and fluorescence microscopy (performed by our sub-contractors).

PROCESSING OF SEWAGE INFLUENT SAMPLES.

Grab samples of sewage influent (1L) were passed through muslin in order to remove large particulates, concentrated by centrifugation (1500 **g**, 15 min) to a volume of 10 mL and subjected to sucrose density flotation (sp.gr. 1.18). The interface was removed, washed three times in grade 1 water and concentrated to a minimal volume (usually between 0.5-2 mL). The pellet was subjected to water-ether concentration and then concentrated to a minimal volume (usually between 0.5-2 mL). For each raw sewage sample, concentrates from both the sucrose interface and the ether pellet were examined for the presence of *Cryptosporidium* sp. oocysts and *Giardia* sp. cysts.

PROCESSING OF SEWAGE EFFLUENT SAMPLES.

Grab samples of sewage effluents (1L) were passed through muslin in order to remove large particulates and then concentrated by centrifugation (1500 **g**, 15 min) to a minimal volume (usually between 0.5-1.2 mL). An aliquot (10%) of each sample was labelled with either anti-*Cryptosporidium* sp. FITC-mAb (Waterborne Inc., LA, USA) or anti-*Giardia* sp. FITC-mAb (Waterborne Inc., LA, USA) and examined by fluorescence microscopy.

EVALUATION OF TWO METHODS FOR RECOVERY OF *CRYPTOSPORIDIUM* SP. OOCYSTS FROM SEWAGE SLUDGE.

A 1 mL sub-sample of sewage sludge, previously determined to be negative for *Cryptosporidium* sp. oocysts, was dispensed into each of eight 50mL centrifuge tubes. A 5 mL sub-sample of oocyst-negative sewage sludge was dispensed into each of four 50mL centrifuge tubes.

Each sludge sample was seeded with 1.45×10^5 *C. parvum* oocysts, the sample volume was adjusted to 50 mL with grade 1 water and then each sample was vortexed to ensure that the *C. parvum* oocysts were suspended.

a). Water-ether concentration.

The liquid from four of the eight one mL oocyst-seeded sewage sludge samples and the four five mL samples was sieved through an Endecott 355 µm (aperture size) brass sieve into a beaker. The filtrate was centrifuged (1050 **g**, for 5 min), the fluid aspirated down to 10 mL and subjected to the water-ether concentration procedure as described

by Bukhari and Smith (1995). Briefly, 2 mL diethyl-ether were added to the concentrated samples and the samples vortexed for 20 sec and then centrifuged (1050 g, 5 min). Both the fat layer and supernatant layers were discarded and the pellet was resuspended in 50 mL grade 1 water and centrifuged (1050 g, 5 min). This washing procedure was repeated twice and the pellet resuspended, in grade 1 water. to a minimal final volume of typically 1 mL.

b). Unconcentrated sludge samples.

The remaining four of the eight one mL oocyst-seeded sludge samples were suspended by vortexing, allowed to settle for I min and a 100 µl aliquot of the supernatant from each sample was placed on a multispot glass slide, air-dried, methanol-fixed and labelled with the anti-*Cryptosporidium* sp. FITC-mAb.

PROCESSING OF SEWAGE SLUDGE.

Five sites, where sewage sludge is disposed of to land, were identified (Table 1) and, for each site, grab samples (approximately 50 mL) of sludge were collected on three separate occasions. A sub-sample (1.0 mL) of each sewage sludge sample was subjected to the water-ether concentration procedure and a 10% aliquot of each concentrated sludge sample was examined for the presence of *Cryptosporidium* sp. oocysts and *Giardia* sp. cysts.

FLUOROGENIC VITAL DYE ASSAY.

An aliquot (10%) of concentrated sewage samples was acidified by incubation in acidified HBSS (pH 2.75, lh, 37°C). Each sample was washed (x3) with HBSS (pH 7.2), concentrated (12,500 g, 30 sec) to 100 µl and 10 µl of 2 mg mL^{-1} 4'6-diamidino-2-phenyl indole (DAPI) and 10 µl of 1 mg mL^{-1} propidium iodide (PI) were added. Samples containing the dyes were incubated at 37°C for 2 h. Optimally diluted anti-*Cryptosporidium* sp. FITC-mAb was added after 90 min incubation with the two dyes and the samples were incubated (37°C) for a further 30 min. Following 2 h incubation with the dyes, each sample was washed (x3) with HBSS (pH 7.2). concentrated to 100 µl and 10µl aliquots were placed on glass microscope slides, covered with coverslips and exammed by fluorescence microscopy.

MICROSCOPY.

Microscopy was performed (x 20 objective and x 12.5 eyepieces) on an Olympus BH2 fluorescence microscope, equipped with Nomarski DIC optics. A blue filter (480 nm-excitation. 520 nm-emission) was utilised for the detection and enumeration of FITC-mAb labelled *Cryptosporidium* sp. oocysts and *Giardia* sp. cysts.

The fluorogenic vital dye assay of Campbell et al., (1992) was utilised for assessment *of Cryptosporidium* sp. oocyst viability using a ***W*** filter block (350 nm-excitation, 450 mn-emission) for DAPI and a green filter block (535 mn-excitation, >610 nm-emission)
for PI.

FLOW CYTOMETRY.

An aliquot (10%) of each concentrated sewage influent and effluent sample was placed in a flow cytometer sample tube, an equal volume of both 10% bovine serum albumin (BSA) and FITC-mAb (anti-*Cryptosporidium* sp./anti-*Giardia* sp.) were

added. The samples were incubated (37°C. 30 min) and then subjected to flow cytometry (Coulter EPICS Elite flow cytometer). The material sorted by the flow cytometer was examined by fluorescence microscopy to enumerate *Cryptosporidium* sp. oocysts and *Giardia* sp. cysts. No sludge samples were analysed by flow cytometry.

STATISTICAL ANALYSIS.

For the seven sewage treatment works. the SPSS for Windows statistical package was used to perform one-way analysis of variance tests (ANOVA) on sewage influents and sewage effluents. For each sewage treatment works, comparison of influents with their respective effluents was performed on SPSS with the Mann-Whitney U test.

The Wilcoxon matched pairs signed ranks test was performed to compare data accrued using fluorescence microscopy with data accrued using flow cytometry followed by fluorescence microscopy.

The statistics tests applied in this study were selected following reference to Statistics for Ornithologists (Fowler, J. and Cohen, L. BTO Guide No. 22).

Results.

OCCURRENCE IN SEWAGE INFLUENTS.

a) Occurrence of *Cryptosporidium* sp. oocysts.

Up to 63.6% of the samples contained *Cryptosporidium* sp. oocysts (Table 2). In oocyst positive sewage influent samples. between 10 and 170 oocysts L^{-1} were detected.

One-way ANOVA was used to compare oocyst concentrations in the influents from all of the seven treatment works. With respect to oocyst concentrations, no significant difference was observed between five of the seven treatment works ($p = 0.21$). The remaining two treatment works (treatment works 6 and 7) contained significantly higher numbers of oocysts than the other five however. no significant difference was observed when oocyst concentrations from these two treatment works were compared ($p > 0.05$).

b) Occurrence of *Giardia* sp. cysts.

Giardia sp. cysts were detected in 70% to 90.9% of sewage influent samples from the seven treatment works, at concentrations ranging from 10 to 13600 cysts L^{-1} (Table 2). One-way ANOVA was used to compare cyst concentrations in the influents from these seven treatment works and no significant difference was observed ($p = 0.29$).

OCCURRENCE IN SEWAGE EFFLUENTS.

a) Occurrence of *Cryptosporidium* sp. oocysts.

Cryptosporidium sp. oocysts were detected in 15.4% to 46.6% of sewage effluent samples from the seven treatment works and, in positive samples. between 10 and 60 oocysts L^{-1} were detected (Table 2). One-way ANOVA was used to compare oocyst concentrations in the effluents from these treatment works and no significant difference was observed ($p = 0.49$).

b) Occurrence of *Giardia* sp. cysts.

Giardia sp. cysts were detected in 15.4% to 91 7% of sewage effluent samples from the seven treatment works and. in positive samples. between 10 and 720 cysts L^{-1} were detected (Table 2). One-way ANOVA was used to compare cyst concentrations in the effluents from these treatment works and no significant difference was observed ($p = 0.18$).

Table 2: Detection of *Cryptosporidium* sp. oocysts and *Giardia* sp. cysts in 1L grab samples of sewage from seven treatment works.

Sewage works.	*Cryptosporidium* sp. oocysts L^{-1}.		*Giardia* sp. cysts L^{-1}.	
-	Influent range [% +ve (n)]	Effluent range [% +ve (n)]	Influent range [% +ve (n)]	Effluent range [% +ve (n)]
1.	<10 - 10 [8.3% (12)]	<10 - 50 [22.2% (18)]	<10 - 1240 [83.3% (12)]	<10 - 210 [72.2% (18)]
2.	<10 - 30 [21.4% (14)]	<10 - 60 [46.6% (15)]	<10 - 480 [85.7% (14)]	<10 - 40 [53.3% (15)]
3.	<10 [0% (11)]	<10 - 20 [27.3% (11)]	<10 - 13600 [90.9% (11)]	<10 - 720 [54.5% (11)]
4.	<10 - 10 [16.7% (12)]	<10 - 20 [16.7% (12)]	<10 - 555 [75.0% (12)]	<10 - 50 [91.7% (12)]
5.	<10 - 20 [40% (10)]	<10 - 20 [15.4% (13)]	<10 - 210 [70.0% (10)]	<10 - 435 [15.4% (13)]
6.	<10 - 170 [63.6% (11)]	<10 - 20 [15.4% (13)]	<10 - 390 [80 0% (10)]	[46.2% (13)]
7.	<10 - 40 [45.5% (11)]	<10 - 60 [33.3% (12)]	<10 - 80 I [72.7% (11)]	<10 - 6 0 [66.7% (12)]

COMPARISON OF THE NUMBERS OF OOCYSTS AND CYSTS IN SEWAGE INFLUENTS AND THEIR RESPECTIVE EFFLUENTS.

a) Occurrence of *Cryptosporidium* **sp.** oocysts.

Sewage effluents from four treatment works (treatment works 1-4) contained higher oocyst concentrations than their respective influents (Table 3). When oocyst concentrations in the influents and their respective effluents were compared statistically. no significant difference was identified ($p = 0\ 27$, $p = 0.18$. $p = 0$. I and $p = 0.93$ respectively). Effluenr samples from two sewage treatment works (treatment works 5 and 7) contained lower numbers of oocysrs than their respective influents (oocyst removal of 54% and 46 4% respectively). Effluent samples from one sewage treatment works (treatment works 6) contained significantly lower ($p = 0\ 0$ I) numbers of oocysts than it's respectve influent samples (93% removal of oocysts)

b) Occurrence of *Giardia* sp. cysts.

Lower cyst concentrations were always found in sewage influents when they were compared with their respective influents. With the exceptions of sewage treatment works 1, 5 and 7, significantly lower ($p < 0.05$) numbers of cysts were detected in sewage effluents than sewage influents. Data from sewage treatment works 2. 3. 4 and 6 indicated that cyst removal ranged between 88.5 - 93.8% (Table 3).

Table 3: Occurrence and removal of *Cryptosporidium* SD. oocvsts and *Giardia* sp. cysts in seven sewage treatment works from England.

Sewage treatment works.	Mean oocyst (cyst) concentration L^{-1} of sewage influent ± SD*.	Mean oocyst (cyst) concentration L^{-1} of sewage effluent ± SD.	Percentage removal of oocysts (cysts).
1.	0.83 ± 2.9 (180.0 ± 345.0)	6.4 ± 14.4 (50 9 ± 72.5)	N/A*(71.7)
2.	3.6 ± 8.1 (160 7 ± 200 4)	8.7 ± 15.0 (10.0 ± 12.0)	N/A*(93.8)
3.	0.0 (1447 7± 4038.4)	3.1 ± 6.2 (94 4± 201.9)	N/A*(93.5)
4.	1.7 ± 3.9 (188.3 ± 164.3)	2.5 ± 6.2 (21.7 ± 15.2)	N/A*(88.5)
5.	5 ± 7.1 (51 0 ± 73.2)	2.3 ± 6.0 (34.2 ±120.4)	54.0 (33.0)
6.	35.5 ± 54.8 (117.3 ± 155 I)	2.5 ± 6.2 (8.3 ± 14 7)	93.0 (93.0)
7.	15.5 f23.8 (19.1 k23.4)	8.3 ± 17.5 (14.2 ± 19 8)	46.4 (26.0)

*N/A = Not applicable. SD = Standard deviation.

DETECTIONOF *CRYPTOSPORIDIUM* SP. OOCYSTS AND *GIARDIA* SP. CYSTS IN SEWAGE SLUDGE.

Recovery of C. parvum oocysts seeded into sewage sludge.

A known number of *C. parvum* oocysts (1.45 x IO'), of a pre-determined viability (20%). was seeded into sewage sludge and subjected to three different procedures, The data accrued (Table 4) indicated that ail three procedures yielded low oocyst recoveries (< 10%) however, for the procedures which were evaluated. our results indicate that the water-ether concentration procedure performed upon 1 mL samples of sewage sludge yielded consistently higher recoveries of *C. parvum* oocysts (Table 4).

Table 4: Recovery of *Cryptosporidium* SD. oocvsts from seeded sewage sludge.

Sludge volume (mL)	Procedure	Mean % recovery ± SD
1.0	water-ether	6.8% ± 0.4%
5.0	water-ether	1.7% ± 0.9%
1.0	suspended in 50 mL	2.0% ± 1 1%

Detection/ enumeration of Cryptosporidium sp. oocysts in sewage sludge.

Three samples of sewage sludge were collected from each of the five sludge disposal sites. Only in one of the five disposal sites were oocysts detected. A mean of 6700

Cryptosporidium sp. oocysts per litre of sludge was detected in three samples from this site. (Table 5).

Detection enumeration of Giardia sp. cysts in sewage sludge.

Three samples of sewage sludge were collected from each of the five sludge disposal sites and all five sites contained either two or three samples that were positive for *Giardia* sp. cysts. The mean cyst concentrations ranged between **6.7** x 10^3 and 1.7 x 10' cysts per litre in these five sites (Table 5) and a statistical comparison of cyst numbers present in each of these sites indicated that only in one site (site 5) were *Giardia* cysts present in concentrations which were significantly higher ($p = 0.01$) than those found in the other sites.

Table 5: Detection of *Cryptosporidium* sp. oocysts and *Giardia* SD. cysts from five sites where sludge is disposed of to land.

Sewage sludge	Mean *no.* of *Cryptosporidium* sp. oocysts detected L^{-1} ±SD (range).	Mean no. of *Giardia sp.* cysts detected L^{-1} ±SD (range).
SS1	**6700 ± 5800 (<10000 - 10000)**	**13300 ± 5800 (< 10000 - 20000)**
ss2	**ND***	**6700 ± 11500 (< I0000 - 20000)**
SS3	**ND***	**23300 ± 20800 (<10000 - 40000)**
SS4	**ND***	**16700 ± 20800 (< 10000 - 40000)**
SS5	ND*	**170000 ± 105000 (50000 -250000)**

***ND = No oocysts detected.**

Table 6: Detection of *Cryptosporidium* SD. oocysts and *Giardia* SD. cysts bv microscoov and flow cytometry.

	Influents		Effluents	
Mean concentrations of:	**Microscopy**	**Flow cytometer**	**Microscopy**	**Flow cytometer**
Cryptosporidium sp. oocysts	8.9 ± 24.5	16.3 ± 54.4	4.6 ± 10.7	3.0 ± 9.6
Giardia sp. cysts	169.8 ± 294.0	209.0 ± 313.0	18.5 ± 38.0	15.4 ± 46.4

DISCUSSION.

In this investigation 8 1 sewage influent samples, from 7 sewage treatment works, were examined for the presence of both *Cryptosporidium* sp. oocysts and *Giardia* sp. cysts and 22 of these samples (27.2%) were positive for *Cryptosporidium* sp. oocysts whereas 63 of these samples (77.8%) were positive for *Giardia* sp. cysts. Concentrations ranging between 10 and 170 oocysts L^{-1} and 10 and 13600 cysts L^{-1} were recorded for *Cryptosporidium* and *Giardia*. respectively. With respect to individual sewage treatment works. the percentage of influents positive for *Cryptosporidium* sp. oocysts ranged from 8.3% to 63.6% whilst the percentage of influents positive for *Giardia* sp. cysts ranged from 70% to 90.9%. These findings support the results of the Scottish study. which also identified that *Giardia* sp. cysts were detected more frequently and at higher concentrations than *Cryptosporidium* sp. oocysts in sewage intluents (Robertson *et al.*, in preparation).
It is likely that both the percentage of samples positive for oocysts and cysts and the number of (oo)cysts detected in positive samples are under-estimates however, recovery efficiencies of methods used for the isolation and enumeration of oocysts and cysts are seldom quoted. Robertson et *al.*, (in preparation) cite a mean oocyst recovery range of 44 - 61% and a mean cyst recovery range of 48 - 52% when sewage influents were seeded with either purified *C. parvum* oocysts of bovine origin or purified *Giardia duodenalis* cysts of human origin and concentrated using the combined sucrose/ether method. In general. concentrated sewage intluents are turbid, and the increase in contaminating particulates exerts not only a detrimental effect on (oo)cyst recoveries, but also interferes with (oo)cysts detection. Whilst analysis of a larger equivalent volume may have revealed further "low level" positive samples, greater commitment to this component of the work would have resulted in fewer samples being analysed.

An equivalent aliquot (10%) of the same concentrated sewage influent and effluent samples was analysed both by Huorescence microscopy and flow cytometry With respect to sewage influents, higher mean numbers of oocysts and cysts were detected using flow cytometry followed by tluorescence microscopy than using tluorescence microscopy alone. With respect to sewage effluents, higher mean numbers of oocysts and cysts were detected by fluorescence microscopy alone than with flow cytometry followed by fluorescence microscopy. In a proficiency scheme both tluorescence microscopy alone and flow cytometry followed by fluorescence microscopy detected similar numbers of oocysts, whereas cytometry performed better than microscopy alone for dirtier samples (Watkins *et al.*, 1995). Another study examined five sewage samples and noted the presence of 3 to 22 fluorescent particles by flow cytometry Subsequent analysis of these samples, by fluorescence microscopy only confirmed oocyst presence in the two samples containing higher numbers (n = 16 and n = 22) of particles (Vesey *et* al., 1991). In neither of these studies were the conclusions based upon statistical comparisons of data. In this study. statistical analyses of over 150 samples indicated that no significant difference existed between these two techniques for detecting either *Cryptosporidium* sp. oocysts or *Giardia* sp. cysts (Table 6).

Despite the limitations of current (oo)cyst concentration and detection techniques, the utilisation of the same isolation, purification and enumeration procedures enabled a

comparison to be made of oocyst and cyst concentrations in seven English sewage treatment works and oocyst and cyst concentrations in six Scottish sewage treatment works. Our data indicate that considerably higher numbers of both oocysts and cysts were detected in samples from the Scottish sew e treatment works. The concentrations of *Cryptosporidium* sp. oocysts and *irdia* sp. cysts in sewage influents are dependent upon both the size of the c ibuting community and the levels of infection and disease within that commur With respect to *Giardia intestinalis* infections, asymptomatic cyst excretors can oe the largest contributor of human-derived cysts into sewage treatment works. lowever. the concept of asymptomatic oocyst excretion in *Cryptosporidium* infe ion of human beings and domestic livestock has not been accepted as readily. Routine diagnostic methods used for the detection of oocysts in faecal samples are inefftcient. For example, the threshold of detection of oocysts in a positive sample is between 10^4 and 10^6 oocysts seeded into 1 gram of faeces (Weber et *al.*, 1991; Webster et *al.*, 1996). By extrapolation, it is possible that the Scottish commumties served by the sewage treatment works studied were either larger and/or had higher levels of *Cryptosporidium* and *Giardia* infecttons and/or disease than their English counterparts.

In contrast to our occurrence data on sewage influent, Rose et *al.*, (1988) reported higher concentrattons of *Cryptosporidium* sp. oocysts (a mean of 5 191 L^{-1}) than *Giardia* sp. cysts (a mean of 5 1 L") in Arizona. USA. However. Rose noted in a later publication that the antibody used for detecting oocysts also cross-reacted with a yeast and that the substitution of this antibody with another more specific antibody reduced their previous oocyst concentrations by two logs (Rose and Botzenhart, 1990). A further, formal, possibility which could account for the differences in these two studies is that a greater proportion of the population which contributed to the sewage influent were infected in the Rose *et al.*, (1988) study. Investigation of the numbers of infected contrtbutors who contribute (oo)cyst-contaminated faeces into a particular sewage treatment works could provide significant data and. if the removal efficiency of the works was known. could be used to determme the times when the greatest likelihood of finding (oo)cysts in effluent discharges might occur.

Not only can the imm une status of the contrtbutors affect cyst numbers. but also. according to Gassmann and Schwanzbrod, (199 1), the time of sample collection may affect cyst concentrations. In their study, in Nancy. France, tr y noted that significantly higher concentrations of *Giardia* cysts were detected when samples were collected at 10.00 a.m. than when they were collected at other hourly intervals between 8.00 a.m. and 7.00 p.m. (Gassmann and Schwanzbrod, 1991). These investigators associated the peaks in cysts concentrattons with "modifications of human activity" which we presume to mean that the morning peak in cyst concentration correlates positively with sewage strength. In our Scottish study, analysis of the data based upon collections made at hourly intervals over 24 h periods failed to corroborate the results of Gassmann and Schwartzbrod (1991) either for cysts of *Giardia* or oocysts of *Cryptosporidium*.

Of the 94 sewage effluent samples examined for the presence of both *Cryptosporidium* oocysts and *Giardia* cysts, 24 (25.5%) contained oocysts whereas 54 (57.4%) contained cysts. Concentrations ranging from 10 to 60 oocysts L^{-1} and 10 to 720 cysts L^{-1} were recorded for *Cryptosporidium* and *Giardia*, respectively. Rose *et al.*, (1988) filtered large volumes of chlorinated secondary sewage effluents (121-757L) and

detected higher concentrations *of* *Cryptosporidium* sp. oocysts (143- 3699 L^{-1}) than *Giardia* sp. cysts (O-2.6 L^{-1}). The lack of specificity of the anti-*Cryptosporidium* antibody used (cited in Rose and Botzenhart. 1990) probably also resulted in over-estimates of oocyst numbers in these chlorinated secondary sewage effluents. In contrast to this American study, earlier investigations in the UK reported concentrations from 0.024 to 38 oocysts L^{-1} in sewage effluent samples (Parker *et al.*, 1993; Carrington and Gray, 1993) Both these UK studies collected samples by large volume filtration and recovered oocysts by flotation methods which are less efficient than the methods used in this study. Our occurrence data from sewage effluents support the data accrued from the recent Scottish study (Robertson *et al*: in preparation) in that *Giardia* cyst concentrations were higher than *Cryptosporidium* oocyst concentrations. Comparison of the occurrence data accrued from both this study and the Scottish study indicates that higher concentrations of both *Cryptosporidium* and *Giardia* were detected in the Scottish study, with oocyst concentrations from <10 to 1000 L^{-1} and cyst concentrations from <10 to 7600 L^{-1} being reported in the Scottish survey.

Of the seven sewage treatment plants studied, four had higher oocyst concentrations in their effluents than their respective intluents, however these differences were not statistically significant. These differences could have occurred because the influent samples and effluent samples were not time matched. As likely is the possibility that higher oocyst recoveries were obtained from effluent samples (which were concentrated by centrifugation only) than from influent samples (which were concentrated by the combined sucrose/ether concentration method). Higher recoveries have been illustrated in the Scottish study where 81-85% of *Cryptosporidium* sp. oocysts seeded into effluent samples were recovered by centrifugation alone, whereas 32-33% of the seeded oocysts were recovered following the combined sucrose/ether method (Robertson *et al.*, in preparation). Furthermore sewage influents contain higher concentrations of contaminating particulates than sewage effluents therefore. interference with oocyst detectron by microscopy by occluding debris. is more likely to occur in influent concentrates. In the remaining three sewage treatment works. higher mean concentrations of oocysts were detected in the influents than in effluents. and oocyst removal efficiencies were highly variable. ranging from 46.4 to 93% Two of the three sewage works utilised both secondary (activated sludge) and tertiary treatment (either rapid sand filtration or microstrainers) and demonstrated oocyst removal efficiencies of 46.4% to 54%. whereas the third sewage treatment works. which employed secondary treatment alone (percolating filters), removed significantly higher numbers of oocysts (removal efficiency of 93%) than the other two sewage works. With respect to *Giardia* cysts, all seven sewage treatment works had higher mean cyst concentrations in the intluents than their effluents and the percentage removal of cysts, which was highly variable, was calculated to range from 26 to 93.8%. Four sewage treatment works demonstrated significant removal of *Giardia* cysts and whilst two of these four sewage works employed secondary treatment only (either a combination of surface aeration' surface filtration or biological filtration), the other two sewage works employed both secondary (biological filtration) and tertiary (either sand filtration or lagoons) treatment. In the remaining three sewage works cyst removal efficiencies were considerably lower (26% to 71.7%) and these sewage works employed either secondary treatment alone (activated sludge) or both secondary (activated sludge) and tertiary treatment (either rapid sand filtration or microstrainers).

Influent samples from two sewage treatment works (treatment works 6 and treatment works 7) contained significantly higher numbers of *Cryptosporidium* sp. oocysts than influent samples from the other live treatment works. As treatment works 6 and treatment works 7 received mainly rural, and mainly rural/farm trade effluents respectively, it is possible that oocysts detected in these two sewage treatment works were derived from animal sources. *Cryptosporidium* sp. infections occur frequently in neonatal livestock and our recent studies indicate that agricultural effluents can contain large numbers of oocysts [Bukhari. 1995; Bukhari and Smith, (in preparation)],

In order to assess the public health significance of oocysts which were recovered from concentrates of sewage influents and effluents, we **assessed** their viability Usually small numbers of oocysts were detected in the 10% equivalent volumes analysed and frequently no oocysts were recovered when a similar proportion of a sample was subjected to further analysis using the fluorogenic vital dye assay. When oocysts were recovered, we considered their numbers to be insufficient (less than three oocysts) to provide an indication of the viability of the oocyst population. On two separate occasions l-2 viable oocysts were identified in two different sewage effluent concentrates. Our viability **data** complement the data from two other Scottish studies (Parker *et* al., 1993: Robertson *et al*; in preparation) and indicate that viable oocysts can be released in sewage effluent discharges.

The potential for introducing oocysts and cysts indirectly into water courses, following the disposal of sewage sludge onto land was also assessed. Sewage sludges from five disposal sites were analysed for the presence of both *Cryptosporidium* sp. oocysts and *Giardia* sp. cysts. *Cryptosporidium* sp. oocysts were detected on two of three occasions from one site only (Table 5). Interestingly, no oocysts were detected in sludge samples (SS3) from treatment works 7 although. firstly, the influent samples from this treatment works contained significantly higher numbers of oocvsts than influent samples from treatment works 1,2,3,4 and 5 and secondly, during the study period. treatment works 7 had a calculated oocyst removal efficiency of 46% It is likely that those sewage sludge samples in which no oocysts were detected probably contained oocysts at concentrations which were below the threshold of detection.

Giardia sp. cysts were detected in sludge samples from all five sites on at least one occasion and cyst concentrations ranged from 1x10' to $2.5x10^5$ L^{-1} Similar cyst concentrations were detected in **sludge** samples from both sewage treatment works located in Scotland ($1x10^3$- $3.3x10^6$) (Robertson *et al.*, in preparation) and sewage treatment works located in the USA (range 70 cysts L^{-1} to $4.14x10^6$ cysts L^{-1}) (Sykora *et* al.. 1991: Jakubowski et al.. 1991; Soares *et* al.. 1994).

Attachment of oocysts and cysts onto other particulates and their subsequent sedimentation causes their accumulation in sewage sludge therefore. the disposal of sewage sludge onto land **could** present the risk of further environmental contamination with viable (oo)cysts. In order to reduce the risk of such environmental contamination. the Department of the Environment's code of practice recommends that under no circumstances should sewage sludge be applied, by whatever method, to areas with a high risk of water pollution. The rates of surface application and soil injection of sewage sludge, identified in the code of practice, are not to exceed 50 m^3 ha^{-1} (4500 gallons. acre-') **and** 140 m^3 ha" respectively and repeat applications of sewage **sludge** are not **be** made within 3 weeks. These regulations prohibit the spreading of sewage sludge on soils with a pH less than 5.0 and also prohibit the application of untreated liquid sewage sludge to pasture except **by** soil injection.

In experimental investigations. mesophilic sludge treatment (37°C) was reported to inactivate >99.9% of *Giardia muris* (which were used to model *G. duodenalis*) cysts within 18-20.5 h (Gavaghan *et al.*, 1993, Van Praagh *et al.*, 1993).

Two published reports on the effectiveness of sludge treatment processes in inactivating *C. parvum* oocysts present contrastmg data. In one study. 99.9% of *C. parvum* oocysts seeded into sludge and subjected to anaerobic digestion for 24 h were Inactivated at 37°C (Stadterman *et al.*, 1995). whereas, in the other study, the data indicated that mesophilic sludge treatment (35°C) of *C. parvum* oocysts required between 24 h and several days to inactivate oocysts (Whitmore and Robertson, 1995). Further investigations are required to assess the significance of these findings.

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