Biol Fertil Soils (1996) 21:30–36

ORIGINAL PAPER

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Movement of the protozoan pathogen *Cryptosporidium parvum* through three contrasting soil types

Received: 1 June 1994

Abstract The potential for transfer of the protozoan pathogen *Cryptosporidium parvum* through soil to land drains and, subsequently, water courses following the application of livestock waste to land was monitored in the laboratory using simulated rainfall and intact soil cores. Following irrigation over a 21-day period, *Cryptosporidium parvum* oocysts applied to the surface of soil cores (initial inoculum concentration 1×10^8 oocysts core^{-1}) were detected, albeit in low numbers, in the leachates from clay loam and silty loam soils but not in that from a loamy sand soil. Variations in leaching patterns were recorded between replicate cores. At the end of the study soil cores were destructively sampled to establish the location of oocysts remaining within the soil. Distribution within cores was similar in all three soil types. The majority ($72.8\pm5.2\%$) of oocysts were found in the top 2 cm of soil, with numbers decreasing with increasing depth to $13.2\pm2.8\%$, $8.39\pm1.4\%$, and $5.36\pm1.4\%$ at depths of 10, 20, and 30 cm, respectively.

Key words *Cryptosporidium parvum* · Livestock waste · Pathogens · Oocysts · Soil cores · Drinking water

Introduction

Livestock wastes are considered a pollution threat because of their high biological O_2 demand and their ability to release nitrates and phosphates to the aqueous environment (Khaleel et al. 1980; Kandeler and Eder 1993). These wastes also contain large numbers of microorganisms which may include pathogenic species (Elliott and Ellis 1977; Strauch 1987; Kearney et al. 1993) and it is now recognized that following the application of livestock waste to land there is a potential for the transfer of these microorganisms to the human population following contamination of plants, soil, and subsequently, water courses.

In the well developed countries of Western Europe and North America with drinking-water treatment facilities designed essentially for bacterial removal, the incidence of large scale waterborne bacterial gastroenteritis is now rare. However, over the last two decades there has been increasing concern over the emergence of 'new' forms of enteritis caused by protozoan parasites and enteroviruses (West 1991). One such disease is cryptosporidiosis caused by the protozoan parasite *Cryptosporidium parvum*, and the waterborne transmission of the pathogen is now well documented (Sterling 1990). This organism is of particular concern since as few as 10 infective oocysts may be required to cause infection (Smith 1992) and because its transmissive oocyst is resistant to current methods used in drinking-water treatment (West 1991).

Cryptosporidiosis is not a new disease. *Cryptosporidium muris* was first described in 1907 and *Cryptosporidium parvum*, the species responsible for the infection of livestock and man, was classified as long ago as 1912 (Tyzzer 1907, 1912, cited in Fayer et al. 1990). However, the importance of the microorganism as a causative agent of scouring in young cattle was only recognized in the 1970s (Current 1987) and it is only through improvements in detection methods and increases in epidemiological surveillance that an indication of the extent of both human and animal infection has now been realized.

Infection is via the faecal-oral route and occurs following ingestion of the transmissive oocyst (4–6 μm in diameter) which is excreted in large numbers (up to 1×10^{10} g^{-1}) in the faeces of infected animals (Smith 1992). The severity of the disease is governed by a number of factors including the immunological status of the host. In the human population immunocompromised patients, especially those with AIDS, suffer a severe infection resulting in protracted diarrhoea which is often irreversible, ultimately resulting in death (Grimason et al. 1990). Immunocompetent patients are less severely affected, diarrhoea and abdominal pain being accompanied by a flu-like malaise which generally lasts 7–14 days, although

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general feelings of malaise may persist for up to a month (Smith 1992). Excretion of oocysts commences with the onset of diarrhoea but may persist long after disease symptoms have ceased (Ungar 1990). In animals, infection is almost exclusive to the young (Robertson and Smith 1992), calves being especially susceptible. In humans, infection may occur at any age although young children are more prone because of their lower hygiene levels (Grimason et al. 1990).

Although many studies have been made of the movement of microorganisms in soil (Gannon et al. 1991a, b; Huysman and Verstraete 1993; Paterson et al. 1993) these have largely concentrated on bacteria and, to a lesser extent, viruses and most have been in risk-assessment studies of microbial inoculants. The potential for transfer of microorganisms, particularly protozoa, to water courses, after the application of livestock waste to land has not been addressed to any great extent. The aim of this study was to investigate the transport, with percolating water, of *Cryptosporidium parvum* oocysts through three different soil types.

Materials and methods

Development of methodology for oocyst extraction from soil

Initial studies were undertaken to investigate the efficiency of the method that Campbell et al. (1992) used to extract *Cryptosporidium parvum* oocysts from faecal samples at removing oocysts from soil. Samples of Denbigh clay loam soil (1 g dry weight equivalent) contained within 50-ml centrifuge tubes were inoculated with 5×10^5 oocysts g^{-1} and incubated at 20 °C for up to 14 days. At intervals triplicate samples were removed and oocysts purified according to Campbell et al. (1992).

In subsequent studies attempts were made to improve the recorded recovery efficiencies by using different extraction fluids in combination with a more rigorous physical dispersal technique. Distilled water, phosphate-buffered saline (pH 7.2), 50 m*M* TRIS (BDH, Poole, UK), 50 m*M* TRIS+0.5% (v/v) Tween 80 (BDH, 0.1% (w/v) sodium cholate (Sigma Chemical Co., Poole, UK), and 0.1% (w/v) sodium cholate+iminodiacetic acid chelating resin (Sigma Chemical Co.) were tested as possible extraction solutions. Extraction solution (20 ml) was added to each of three replicate soil samples (as used in the above study) and the contents vortexed for 1 min before centrifugation (10 min, 1500 g; Centaur 1 bench centrifuge, MSE, Crawley, UK). The supernatant fraction was discarded and the washing procedure repeated. After the second wash the pellet was resuspended in 10 ml of the extraction solution, 10 glass beads (3 mm diameter) added, and the sample mixed for 15 min on a wrist-action shaker (Stuarts Scientific flask shaker, BDH; setting 7). The resulting suspension was underlaid with 10 ml cold sucrose solution (specific gravity 1.18) and centrifuged (15 min, 1500 g). The interface (10 ml) was removed to a clean 50-ml centrifuge tube using a syringe fitted with a 0.8×40 mm hypodermic needle (Terumo, Fisons, Loughborough, UK) and washed three times in distilled water. After the final wash the supernatant fraction was removed down to 1 ml, and aliquots were stained and examined microscopically for the presence of oocysts (see below). Samples (20 µl) were dried onto 1-cm^2 areas on microscope slides, fixed in methanol, stained in auramine phenol (Lempert, BDH), counterstained in dilute carbol fuschin (Gurr, BDH), and allowed to dry (Casemore et al. 1985). Slides were examined using an epifluorescence microscope (BH2, Olympus Optical Company, London, UK) equipped with a blue filter block (excitation 490 nm, emission 510 nm). The number of oocysts in each of 100 randomly chosen fields of view was counted, and at least three replicate slides for each sample were examined. Numbers of oocysts detected were expressed (with SE) as the mean of replicates. Student's *t*-test was used to assess the difference between sample means.

Soil columns

Intact soil cores were taken from each soil type by driving 35-cm lengths of 15 cm (inner diameter) polyvinyl chloride (PVC) drain piping 30 cm into the soil. To aid this process, detachable steel cutting edges were fitted to the bottom of each tube. Three contrasting soil types were used in the study, a loamy sand, a silty loam, and a clay loam; further details of these soils are shown in Table 1. All three soils were taken from perennial ryegrass leys and cores were taken with the vegetation intact. Once extracted, the cores were transported back to the laboratory and brought to maximum water-holding capacity by irrigation over a 5-day period. Five replicate cores were mounted on an irrigation/support apparatus in a constant temperature room (18 °C) and *Cryptosporidium parvum* oocysts (supplied by J. Kemp and S. Wright, Moredun Research Institute, Edinburgh, UK) were applied to each core at a density of ca. 1×10^6 oocysts $core^{-1}$. After 16 h, artificial rainwater (Skibber and Cresser 1986) was applied to each core over a 4-h period at a rate of 70 ml h^{-1} by means of a peristaltic pump; 10 individual lines were used to ensure even dispersal of the rainwater over the surface of the core and these were suspended at a height of 5 cm above the top of the core by inserting them through a clear plastic disk mounted over the top of the core. Irrigation was subsequently repeated on alternate days over a period of 21 days. Leachate from each core drained, via a plastic funnel, into a 500 ml Winchester bottle, and was collected for 24 h following the start of each irrigation event and subsequently analyzed for the presence of oocysts. At the end of the 21-day study, cores were destructively sampled and soil removed to determine the location of oocysts remaining within the cores. Soil was taken from the top and bottom of the core (at depths of 1 and 30 cm) before holes (4.5 cm diameter) were drilled through the plastic drain piping at depths of 10 and 20 cm. A soil corer (4.25 cm diameter) inserted through the holes was used to withdraw soil from the whole width of the tube and subsamples of this soil, together with that collected from the top and bottom of the core, were examined individually for the presence of oocysts.

Extraction and concentration of oocysts from leachate and soil samples

The volume of leachate collected from replicate cores after each irrigation event was measured (average for all soil types at all irrigation times 217 ml, SEM 2.01 ml) and then transferred to a 300-ml centrifuge tube. Samples were centrifuged (Europa 24M centrifuge,

Table 1 Characteristics of the three contrasting soil types used in the soil core studies to investigate the vertical transport of the protozoan *Cryptosporidium parvum*

Characteristic	Soil type		
	Loamy sand	Silty loam	Clay loam
Soil series	Bridgenorth	Teme	Denbigh
pH	6.35	5.60	6.09
Sand (%)	79	32	40
Silt (%)	11	52	16
Clay (%)	10	16	29
Organic matter (%)	3.91	5.55	6.10

MSE, Crawley, UK) for 15 min at 1500 g and ca. 175 ml of the supernatant fraction was discarded. The pellet was resuspended in the remaining leachate and transferred to a 50-ml centrifuge tube. The samples were centrifuged again for 15 min (this and all subsequent centrifugation steps were carried out using a MSE Centaur 1 Bench Centrifuge, 1500 g) and the supernatant fraction discarded, leaving ca. 10 ml leachate and the pellet in the tube. This was resuspended in 20 ml 50 m*M* TRIS +0.5% Tween 80, mixed by vortexing, and recentrifuged for 10 min. The supernatant fraction (20 ml) was removed, leaving 10 ml and the pellet remaining in the tube; these were vortexed to mix before the resulting suspension was further concentrated by sucrose gradient centrifugation and washed as for soil samples (see above). After the final wash the supernatant fraction was aspirated down to 1 ml.

To extract oocysts from soil, 1 g dry weight equivalents of soil removed from samples taken from each depth of the five replicate soil cores were weighed into 50-ml centrifuge tubes. Each sample was analysed in duplicate. To each tube 50 m*M* TRIS+0.5% Tween 80 (20 ml) was added and oocysts extracted by the method described above.

Aliquots (20 µl) of oocysts concentrated from leachate or soil were dried (at 37 °C) onto 1-cm^2 areas on microscope slides and stained and examined as detailed above.

Results

The preliminary studies undertaken to evaluate the relevance for soil studies of the technique that Campbell et al. (1992) used to extract oocysts from faecal material showed that modification was needed for use in long-term experimental soil studies. Although 61.6% of the added oocysts were extracted immediately following inoculation, the efficiency of recovery declined rapidly, so that by day 7 only 0.32% of the *Cryptosporidium parvum* oocysts initially inoculated were detected, and by day 9 numbers were below the level of detection (Table 2).

A more rigorous extraction procedure was developed subsequently and the efficiency of the different extraction solutions evaluated. Soil samples that had been inoculated with 5×10^5 oocysts g^{-1} were extracted using the different solutions at regular intervals over a 21-day period. Table 3 shows the numbers of oocysts extracted on day 14, which were typical of the pattern of results obtained throughout the study, although by day 21 numbers of oocysts extracted from the sodium cholate and sodium cholate plus chelating resin samples had fallen to below quantifiable limits. Extraction using 50 m*M* TRIS +0.5% Tween 80 consistently gave significantly higher ($P<0.01$) oocyst recoveries and was therefore used in all subsequent investigations.

Once a method had been developed to extract *Cryptosporidium parvum* oocysts from soil, studies were carried out to investigate the fate of the pathogen following its application to soil. Vertical transport with percolating water of the protozoan was demonstrated in all three soil types although variations in the rate and extent of this movement were measured. Whilst oocysts were detected in the leachate from both the silty loam and the clay loam cores, none were detected in that from loamy sand cores.

In the silty loam soil (Fig. 1) oocysts were detected in leachate from four of the five replicate cores on one or more sample dates over the complete course of the study. The majority (99.1%) of oocysts were detected in the 5 days immediately following introduction and none were detected after day 9. One of the five replicate cores did not release detectable numbers of oocysts over the entire study period. However, on destructive sampling, this non-release core had a distribution of oocysts identical to that observed in the other four replicates, indicating that the eluted oocysts were probably below quantifiable limits.

In the clay loam soil (Fig. 2) oocysts were detected in the leachate of all five replicate cores for the first 7 days after introduction, but thereafter the effluent of only one core continued to contain oocysts and numbers released remained at a similar level over the rest of the 21-day study.

At the end of the study, soil cores were destructively sampled to determine the distribution of oocysts remaining within the cores. Significantly higher numbers of oocysts were recovered from the silty loam soil than from either the loamy sand or clay loam soils (Fig. 3). However, the distribution of oocysts remaining within the cores was similar in all three soil types. The majority of oocysts were located in the top few centimetres of soil (72.8±5.19% at a depth of 1−2 cm), numbers subsequently decreasing with increasing depth with averages for the three soils being 13.2±2.8%, 8.39±1.4%, and 5.36±1.4% at depths of 10, 20, and 30 cm respectively.

Table 2 Effect of incubation time on number of oocysts extracted from Denbigh clay loam soil initially inoculated with 5×10^5 oocysts g^{-1} dry weight. Values are means±SEM (*BQL* below quantifiable limits, $<5.29\times10^2$ oocysts g^{-1} dry weight)

Incubation time	Oocysts extracted ($\times10^3$ g^{-1} dry weight)	Oocysts extracted (% of initial inoculum)
15 min	308±28	61.6
1 h	169±23	33.8
24 h	21.4±2.9	4.3
4 days	27.3±2.54	5.4
7 days	1.59±0.53	0.3
9 days	BQL	BQL

Table 3 Effect of different extraction solutions on numbers of oocysts recovered from Denbigh clay loam soil 14 days after inoculation with 5×10^5 oocysts g^{-1} dry weight. Values are means±SEM; those followed by the same letter are not significantly different ($P<0.01$)

Extraction solution	Oocysts extracted ($\times10^3$ g^{-1} dry weight)
0.1% w/v Sodium cholate	3.3±1.40a
0.1% w/v Sodium cholate + chelating resin	16.2±2.40b
50 m*M* TRIS	30.9±3.51c
Phosphate-buffered saline (pH 7.2)	28.8±3.30c
50 mM TRIS +0.5% v/v Tween 80	76.8±8.01d
Distilled water	31.7±3.70c

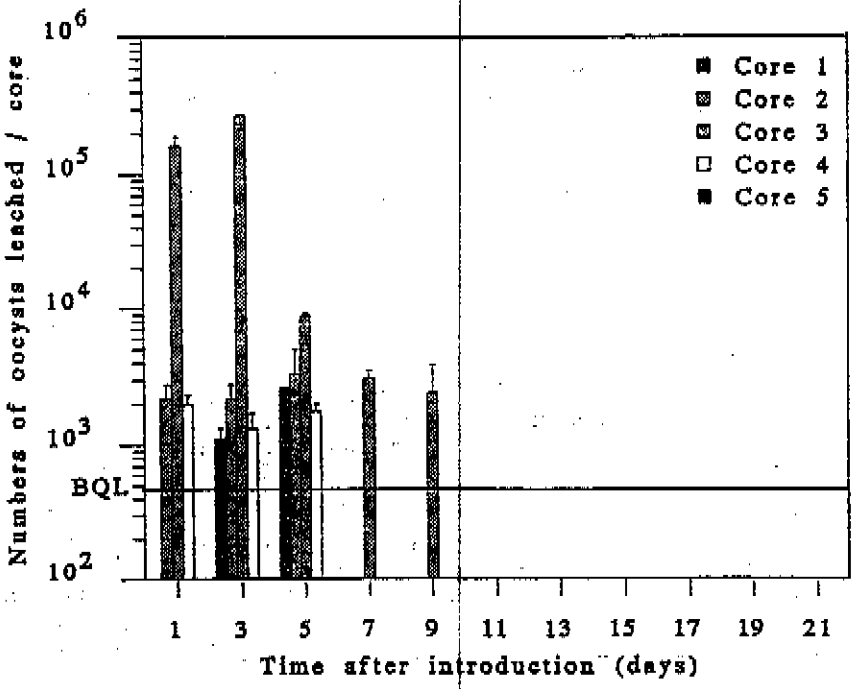

Fig. 1 Leaching pattern of *Cryptosporidium parvum* oocysts from replicate cores of Teme silty loam soil following irrigation on alternate days. *BQL* below quantifiable limits ($< 5.29 \times 10^2$ oocysts). *Vertical bars* represent SEM

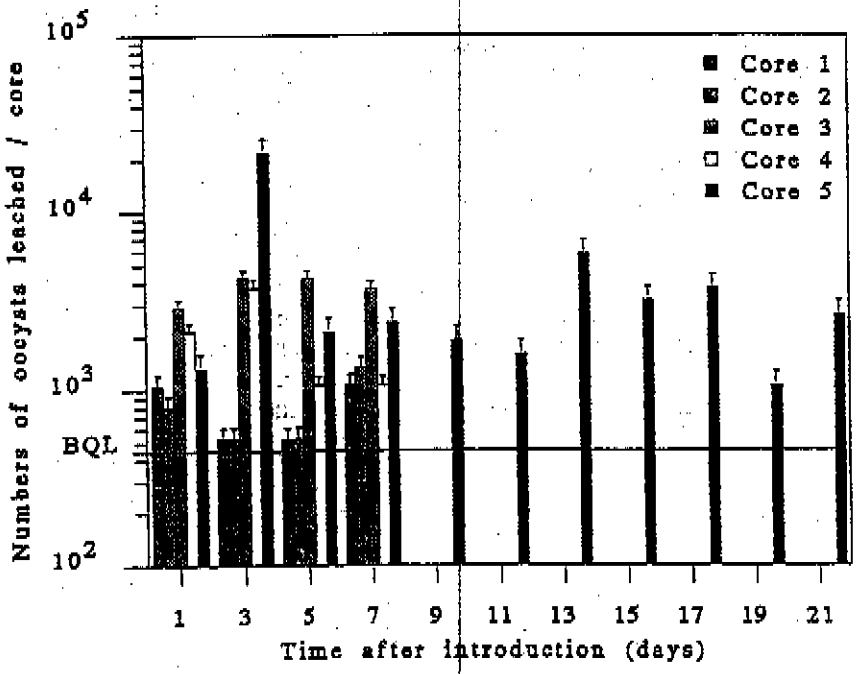

Fig. 2 Leaching pattern of *Cryptosporidium parvum* oocysts from replicate cores of Denbigh clay loam soil following irrigation on alternate days. For other explanations see Fig. 1

Discussion

Many studies have examined the effect of soil type on the movement of both bacteria and viruses in soil, although few, if any, have examined the movement of protozoal cysts. Due to their small size, the major factor influencing viral movement is adsorption to soil particles (Burge and Enkiri 1978) whereas with bacteria, because of their larger size, filtration, and sedimentation will also play a part in governing the degree of microbial movement (Reddy et al. 1981; Gannon et al. 1991 b; Bitton and Harvey 1992; Tan et al. 1992). Protozoal cysts are considerably larger than bacterial cells (*Cryptosporidium parvum* oocysts are 4–6 μm in diameter) and, as with bacteria, filtration and sedimentation in addition to adsorption can be expected to affect the extent of movement. Differences in the degree and rate of movement of bacteria and viruses have often been reported, with most studies indicating increased movement in sand as opposed to soil (Bashan and Levanony 1988), in coarse as opposed to fine soil fractions (Tan et al. 1991), and in sandy as opposed to clay soils (Bitton et al. 1974; Huysman and Verstraete 1993). This variation is due, in part, to differences in the adsorp-

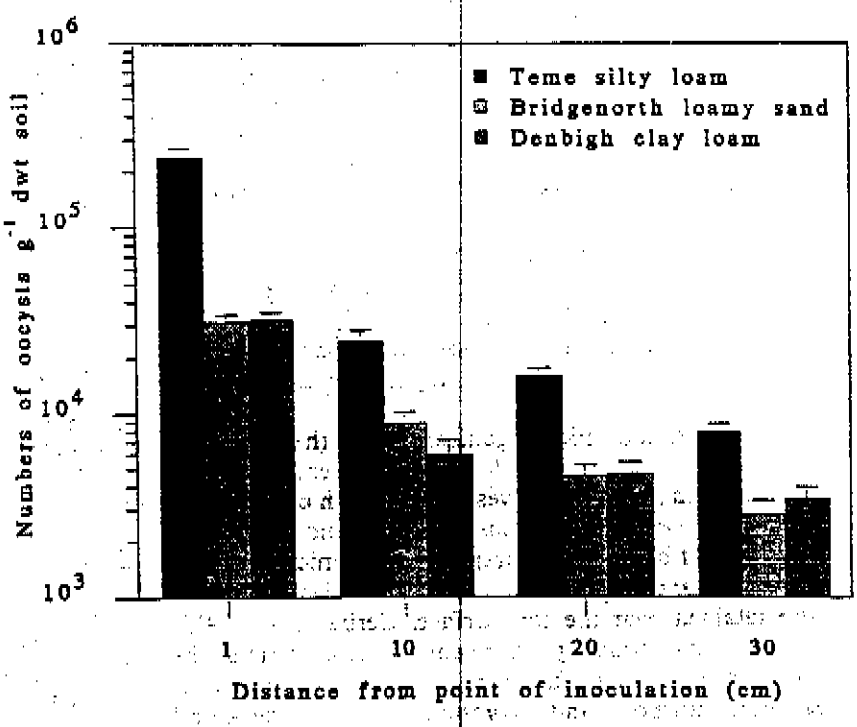

Fig. 3 Distribution of *Cryptosporidium parvum* oocysts in cores of silty loam, clay loam, and loamy sand soil 21 days after introduction and irrigation. Cores were originally inoculated with 1×10^8 oocysts $core^{-1}$. *Vertical bars* represent SEM. *dwt* Dry weight

tive properties of soils and to the number and size of micropores (Griffin and Quail 1968; Postma et al. 1989; Tan et al. 1992). Clay and organic matter are the major soil components affecting adsorption of bacteria and viruses because of their large surface areas and their negative charge (Marshall 1971; Reddy et al. 1981).

In our study, transport of *Cryptosporidium parvum* oocysts through soil and into leachate was greater in a silty loam and a clay loam soil than in a loamy sand soil. A study by Paterson et al. (1993) demonstrated similar results for recoveries of an introduced bacterium, *Pseudomonas fluorescens*, with greater recoveries in the leachate from intact clay loam soil cores than from either sandy loam or loamy sand soil cores. As the majority of evidence suggests that the degree of adsorption is greater and the size of micropores is smaller in clay as opposed to sandy soils, our results indicate that factors other than adsorption and micropore size affected the extent of oocyst movement in the present study. A major difference between our study and those cited above reporting greater movement in coarse sandy soils than in clay soils is that while we used intact soil cores, the other studies were all carried out in disturbed and repacked cores. Although repacked cores provide a homogeneous environment in which to study the effect of individual soil variables on microbial movement (Trevors et al. 1990; Gannon et al. 1991a; van Elsas et al. 1991), intact or undisturbed soil cores are more representative of field conditions (Bentjen et al. 1989).

One of the obvious differences between intact soil cores and those that have been repacked is the lack of natural soil structure and the notable absence of macropores in the latter. Soil water content and the flow of water through soil are major factors affecting the extent of microbial transport in soil (Kuikman et al. 1990; Trevors et al. 1990; van Elsas et al. 1991). While the importance of macropores in water transport through soils has been recognized for many years (Thomas and Phillips 1979), it is only recently that the potential effects on the release of soil chemical pollutants have been considered (White 1985). The rapid flow of water through macropores, thereby largely bypassing the filtering and adsorptive effects of the soil, may also greatly increase the risk of pathogen transport to groundwater and land drains. This hypothesis is supported by many studies indicating that smaller numbers of bacteria are transported through repacked soil cores than through intact cores where channels and pores have been retained (Smith et al. 1985; van Elsas et al. 1991). The presence of macropores may explain the large difference observed in the oocyst flow pattern in one of the Denbigh clay loam cores in the present study although, in general, remarkably good agreement was recorded between replicate soil cores.

Although all three soil types had been inoculated with similar numbers of oocysts, significantly higher numbers were recovered from the silty loam soil than from either the loamy sand or the clay loam soils. From the results of preliminary studies (J. Mawdsley, unpublished observations) it is clear that this was not due to increased extraction efficiencies in the silty loam soil compared to those measured in loamy sand or clay loam soils, as oocysts were more easily extracted from the loamy sand than from either the silty loam or the clay loam soil. Greater recoveries from the loamy sand in these preliminary studies were probably due to differences in the adsorptive properties of the soils. The loamy sand soil had significantly lower organic matter and clay contents than either the silty loam or clay loam soil. Both organic matter and clay play

a major role in the adsorption of microbes in soil, due to their large surface area and negative charge, and many studies have shown increased adsorption in soils with high organic matter or clay contents (Guy and Visser 1979; Bashan and Levanony 1988; Huysman and Verstraete 1993). However, this does not explain the higher recoveries of oocysts from the silty loam cores. Perhaps oocysts in silty loam soils are less prone to predation or are less readily degraded compared to oocysts in clay loam or loamy sand soils. We are at present investigating this phenomenon and studies have already shown differences in the viability of *Cryptosporidium parvum* oocysts in a loamy sand compared to a clay loam soil (Mawdsley 1994).

The retention of microorganisms within the upper sections of soil cores, as recorded in the present study, has been reported in previous investigations with other microorganisms. Wollum and Cassel (1978) monitored the movement of bacteria and streptomycete conidia in a sandy soil and showed that the majority of microorganisms were retained near the soil surface. Gerba et al. (1975) suggested that straining (i.e., retention) of bacteria at the soil surface is a major factor in determining the extent of bacterial transport, and suggested that as bacteria and other particles are strained out at the soil surface, they will in turn have a filtering effect.

The present study has shown that under the conditions examined, with purified oocysts applied in very high numbers, leaching of the pathogen down through the soil profile does occur although the extent of this is affected by soil type. However, the distances examined in this study are shorter than those required for the pathogen to enter land drains. Further studies are needed for an accurate assessment of the real risk of environmental contamination, as the extent and rate of movement through deeper soil horizons may vary significantly from the movement measured in topsoil. It seems likely that a combination of factors, including a natural filtering effect, predation, and loss of viability, may interact to decrease the risk of further environmental contamination following the contamination of soil with oocysts; further studies are in progress to examine these possibilities.

Acknowledgements This work was funded through a MAFF/SOAFD Open Contract, CSA 2064 Protozoan, bacterial and viral pathogens, farm animal wastes and water quality protection. We thank S. Wright and J. Kemp at the Moredun Research Institute, Edinburgh, for the supply of *Cryptosporidium parvum* oocysts, Z. Bukhari and Prof. H. Smith at the Scottish Parasite Diagnostic Laboratory, Glasgow, for instruction on the techniques used in oocyst enumeration, and Drs. B. Pain and M. Theodorou at IGER for useful advice and discussions.

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