Biol Fertil Soils (1996) 23:215–220

ORIGINAL PAPER

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Use of a novel soil tilting table apparatus to demonstrate the horizontal and vertical movement of the protozoan pathogen *Cryptosporidium parvum* in soil

Received: 20 October 1995

Abstract A novel greenhouse based soil tilting table apparatus was used to investigate the potential for movement of the protozoan pathogen *Cryptosporidium parvum* both through and across a low permeability soil following the application of contaminated livestock waste to land. Soil blocks supported at an angle of 7.5% by the soil table were inoculated at one end with oocyst seeded slurry and subsequently irrigated at regular intervals over a 70-day period. Movement of the pathogen in runoff was demonstrated for at least 21 days and in one case in excess of 70 days from the time of inoculation. Water was also lost following percolation down through the soil profile and significant numbers of oocysts were also lost via this route, average numbers leached decreasing from $8.36 \pm 0.56 \times 10^6$ at day 1 to $2.27 \pm 0.73 \times 10^4$ at day 70. At the end of the study cores were removed from the soil blocks to determine the location of oocysts remaining within the soil. Numbers decreased down through the soil profile and as the distance from the point of inoculation increased so that 70 cm from the point of inoculation no oocysts could be detected in the soil at any depth. This implies that oocysts contained in runoff stay in the aqueous phase and do not precipitate out onto the soil surface, suggesting that even if the distances travelled are increased there may still be a significant pollution threat.

Key words *Cryptosporidium parvum* · Livestock waste · Runoff · Pollution · Soil tilting tables · Protozoa · Oocysts · Transfer routes

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Introduction

The application of livestock waste to land is a common practice due to the value of the waste as a fertilizer. However, it is not without environmental risk because of the high BOD (Biological Oxygen Demand) of wastes and their potential to release nitrates and phosphates into the aqueous environment (Khaleel et al. 1980). An additional risk factor which has received less attention is the large numbers of microorganisms, including potentially pathogenic species, that these wastes contain. To date very little research has focused on the spread of pathogens from livestock waste to the human population following the application of livestock waste to land. In order to assess what risks, if any, are associated with this practice the interaction of two major factors must be considered. Firstly the potential for transport of pathogens both through and across soil and secondly the survival characteristics of the pathogen in the environment. Only when both these factors have been considered will an accurate risk assessment be possible. In our study we chose to monitor movement of the protozoan *Cryptosporidium parvum.* This parasite is of particular concern as high numbers of the transmissive oocysts are excreted in the faeces of diseased animals, because the infective dose in livestock and humans is low, and because the pathogen is resistant to the current methods used in drinking water treatment (Smith 1992; Robertson et al. 1994). Very few studies have monitored either the movement or survival of *C. parvum* oocysts in the environment (Harvey et al. 1995; Robertson et al. 1992) and studies in soil are particularly lacking.

Following the application of waste to land the transfer of microorganisms from soil to the aqueous environment may occur via one of two routes, depending on soil type and conditions. On permeable soils the most likely transfer route is down through the soil profile to land drains or ground water. However, if soil is saturated, of a heavy clay type or situated over an impermeable substrate, any water applied may be lost through surface runoff (Moore 1989) or by flow along the impermeable substrate. Runoff from agricultural land after natural rainfall or irrigation of-

ten reaches water courses which are subsequently used as sources for public water supplies. Consequently, when such land has been treated with contaminated animal (or human) wastes the possibility exists for the transmission of pathogens to the human or animal population (Patni et al. 1985). Several large-scale outbreaks of water-borne cryptosporidiosis have occurred in both the UK and the USA, but although water is now recognised as a major transmission route (Robertson et al. 1994) the pathways by which water is initially contaminated may be hard to prove. However, in some cases circumstantial evidence has suggested either direct or indirect contamination from sewage or livestock waste (Anon 1990; Mackenzie et al. 1994). The aim of our study was to investigate to what extent *C. parvum* oocysts applied as a suspension in slurry, to an impermeable soil, are transported both horizontally and vertically following simulated rainfall. A novel soil tilting table approach was developed to simulate field conditions but avoid the possible spread associated with field trials of this class two (ACPD 1990) pathogen into the environment.

Materials and methods

Soil table apparatus: construction and assembly

To simulate field conditions and, in particular, the effect of slope on the horizontal movement of *Cryptosporidium* oocysts in runoff without the risks of environmental contamination associated with field studies, a novel soil support system (tilting table apparatus, Fig. 1) was developed. For this purpose soil blocks were removed from the field intact and supported on a specially constructed table which allowed adjustment of the angle of slope. The system consisted of three individual units mounted upon one support frame. Each unit consisted of a plastic box (internal dimensions: 80 cm long × 56 cm wide × 25 cm deep) with a removable, plastic, perforated false bottom through which leachate ran into a collection vessel via the base of the box. In addition each unit had a detachable lip which was pushed into the top front end of the soil block at a depth of 4 cm and clipped into place to allow collection of runoff, which subsequently drained to a plastic reservoir. Thus, runoff was kept separate from leachate which had percolated down through the soil. Each unit was mounted on a hydraulic jack to allow independent adjustment of the slope.

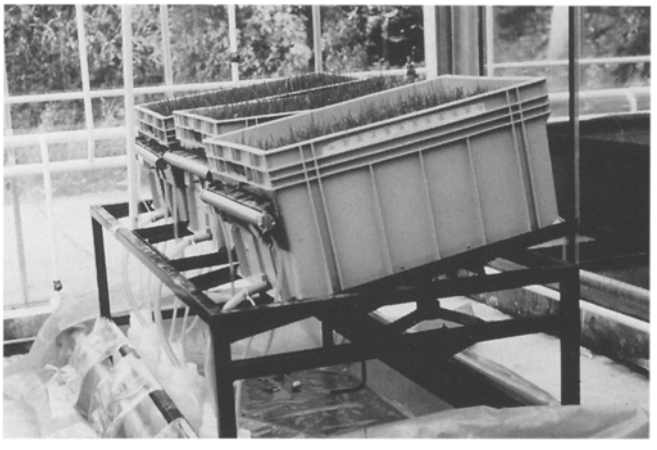

Fig. 1 Soil tilting table apparatus used to monitor the horizontal and vertical movement of *Cryptosporidium* in soil

Soil blocks of 20 cm depth but otherwise similar in dimensions to the boxes detailed above were cut (with vegetation intact) from a perennial ryegrass ley on a poor draining silty clay loam soil (Conway series, pH 6.6, Loss on Ignition 10.3%, 12.3% sand, 54.5% silt, 33.2% clay) using the front-end loader of a tractor. These blocks were then lifted up from the ground, the detachable perforated false bottom from the box slid under the block of soil and the block, together with the false bottom, carefully lowered into the boxes after the soil had been trimmed to fit with a spade. The boxes containing the soil blocks were subsequently mounted upon the support apparatus, which was housed inside a controlled environment greenhouse. The interfaces between the edges of the soil blocks and the boxes were sealed with expanding polystyrene foam to ensure that no water could escape into the collection vessels other than along the intended routes.

The three soil blocks were initially irrigated in a horizontal position over a 2-week period to ensure complete saturation (i.e. until the volume of leachate and runoff collected daily was equivalent to the volume of water applied). The tables were then jacked up so that the surface of each was at a 7.5% angle – this was measured using a spirit level placed on the surface of each block.

Cow slurry (420 cm^3) was inoculated with ca. 5×10^9 *Cryptosporidium parvum* oocysts (supplied by J. Kemp and S. Wright, Moredun Research Institute, Edinburgh) and stored at 4°C for 4 days. This number of oocysts was selected for two reasons. Firstly, so that it was probable that numbers of oocysts in the leachate or runoff would be detectable, and secondly it is possible that this number of oocysts could be applied to fields as infected animals may excrete up to 10^{10} oocysts day^{-1} (Smith 1992). The slurry was subsequently divided into three equal portions of 140 cm^3 and one portion applied as a 5-cm band to the top edge of each block (i.e. at the top of the slope). This rate of application was equivalent to the maximum recommended field application rate of 50 m^3 slurry ha^{-1} (MAFF 1991). After 24 h, 3.4 l water was applied to the surface of each block using a watering can fitted with a perforated tube extending across the width of the block. The water was applied gradually to the entire surface of the block, starting at the top and moving up and down the block at a rate of 3 s per length until all of the water had been applied. The runoff and leachate from each table was then collected for 24 h following irrigation.

Initially, the tables were irrigated with water and sampled twice a week but this sampling frequency was reduced as the experiment progressed. Sampling times were 1, 4, 7, 11, 14, 17, 21, 28 35, 42, 56 and 70 days after the initial application of slurry.

At the end of the study the location of oocysts remaining within the soil blocks was investigated. A corer (4.25 cm diameter) was used to sample the soil. Cores were taken at three positions, 10, 40 and 70 cm from the top edge of the table. At each position soil was taken from three depths, 0–6, 7–13 and 13–20 cm. In each of these nine zones three replicate samples were taken across the width of the table and combined, with thorough mixing, to form a single sample. With three blocks and samples from nine zones in each, a total of 27 samples were available. Aliquots (1 g dry weight equivalent) from each of these 27 samples were analysed in duplicate for the presence of oocysts.

Extraction and concentration of oocysts from leachate and soil samples

The volumes of runoff and leachate collected from each table following each irrigation were measured. Aliquots of the total volume collected for each fraction were then centrifuged (1500 g, MSE Europa 24M centrifuge) until the whole of each sample had been concentrated down to approximately 50 ml. The samples were then transferred to a 50-ml centrifuge tube and centrifuged again for 15 min (this and all subsequent centrifugation steps were carried out using an MSE Centaur 1 Bench Centrifuge, 1500 g) and the supernatant fraction discarded, leaving ca. 10 ml leachate and the pellet in the tube. The pellet was resuspended in 20 ml 50 m*M* TRIS + 0.5% Tween 80, mixed by vortexing and re-centrifuged for 10 min. The supernatant

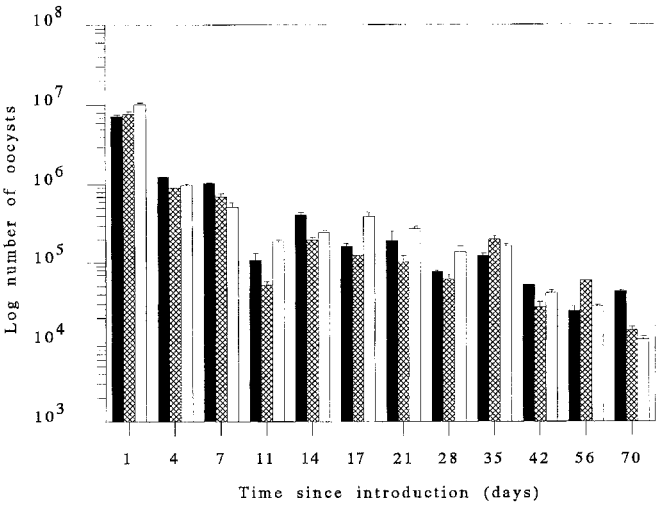

Fig. 2 *Cryptosporidium parvum* oocysts in the leachate from each of the three soil tilting tables following irrigation. *Bars* represent standard errors

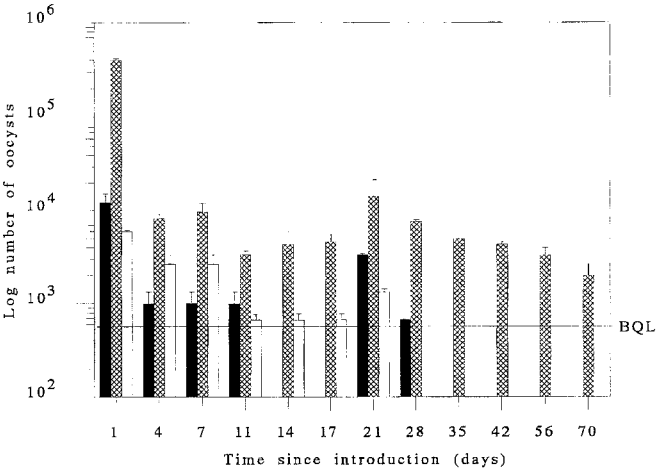

Fig. 3 *Cryptosporidium parvum* oocysts in the runoff from soil tilting tables following irrigation. *BQL*, below quantifiable limits (i.e. $< 5.29\times10^2$ oocysts). *Bars* represent standard errors

fraction (20 ml) was removed, leaving 10 ml and the pellet remaining in the tube. These latter fractions were mixed by vortexing before the resulting suspension was underlaid with 10 ml cold sucrose (1.18 specific gravity) and centrifuged for 15 min. Ten millilitres of the interface was transferred, using a hypodermic syringe, to a clean centrifuge tube and washed 3 times with distilled water, the supernatant fraction being aspirated down to 1 ml after the final wash. Oocyst numbers were estimated in aliquots of this 1 ml.

For the extraction of oocysts from soil, 1-g aliquots of the pooled soil samples from each location on the three replicate tables were weighed into 50-ml centrifuge tubes. Each sample was analysed in duplicate. Oocysts were extracted from soil by the method described by Mawdsley et al. (1996).

For total counts aliquots (20 µl) of concentrated oocysts from soil, leachate and runoff samples were dried onto 1-cm^2 areas on microscope slides at 37°C and oocysts stained with fluorescein isothiocyanate (FITC)-labelled monoclonal antibody prior to enumeration. Twenty microlitres of FITC-conjugated monoclonal antibody (Shield Diagnostics, Dundee, UK) was added to each dried sample and slides were incubated at 37°C in a humid container for 1 h. The slides were then washed 3 times in phosphate-buffered saline (0.2 *M*, pH 7.2) and allowed to dry before being examined using an epifluorescence microscope (BH2, Olympus Optical Company, London) equipped with a blue filter block (excitation 490 nm, emission 510 nm). The numbers of oocysts in each of 100 randomly chosen fields of view under 400 × magnification were counted, and at least three replicate slides for each sample were examined. Numbers of oocysts detected are expressed as the mean of replicates and are shown with standard errors; these are adjusted from numbers / slide to numbers in the volume of leachate / runoff collected. Student's *t*-test was used to assess the difference between sample means.

Results

Oocysts were detected in both the runoff and leachate from soil blocks immediately following the first irrigation event (Figs. 2, 3), and continued to be detected for at least 21 days (in the runoff) or 70 days (in the leachate).

The numbers of oocysts in the leachate from the three replicate soil blocks are shown in Fig. 2. Numbers from all three blocks were very similar at each sample date, and declined steadily over the course of the study so that the average numbers leached from the three soil blocks declined significantly ($P<0.001$) from $8.36 \pm 0.59 \times 10^6$ at day 1 to $2.27 \pm 0.73 \times 10^4$ at day 70.

The numbers of oocysts in the runoff from the three replicate blocks (Fig. 3) showed a greater degree of variation than for those in the leachate samples. Block 2 consistently shed the highest numbers of oocysts; oocysts were lost over the entire 70 days of the study. This may have been related to the fact that this was the block from which the highest volume of water was lost as runoff (Table 1). The numbers of oocysts in runoff from the remaining blocks were lower and consistently below quantifiable limits (i.e.$<5.29\times10^2$) after day 21 and day 28 for blocks 3 and 1, respectively.

Throughout the study significantly ($P<0.01$) higher numbers of oocysts were contained in leachate than in runoff across the soil surface (Fig. 4). Although the relative proportions of oocysts in the runoff and leachate from the different blocks varied, the total numbers lost (in both runoff and leachate) from each of the three blocks over the entire study were not significantly different ($P>0.05$): 1.07×10^7 (block 1), 1.06×10^7 (block 2) and 1.30×10^7 (block 3).

At the end of the study the tables were destructively sampled to determine the location of oocysts retained

Table 1 Volumes of liquid collected as runoff or leachate from individual blocks on the soil tilting table apparatus; values are the means of volumes collected on the 12 sampling dates over the whole study and are shown±standard error

Sample type	Volume of liquid collected (ml)		
	Block 1	Block 2	Block 3
Runoff	203±17	1122±67	410±31
Leachate	2346±74	1391±64	2231±118

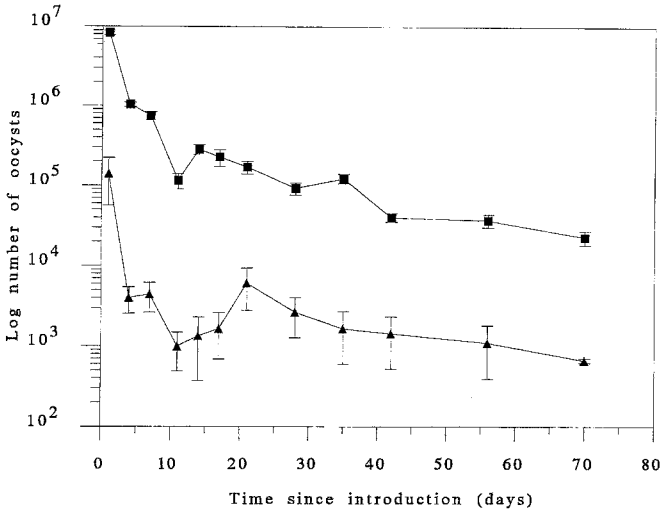

Fig. 4 A comparison of the numbers of *Cryptosporidium parvum* oocysts contained in the runoff (▲) and leachate (■) from the soil tilting tables. Numbers are the average for the three replicate blocks; where one or more values were below quantifiable limits they were taken as zero in the calculations. *Bars* represent standard errors

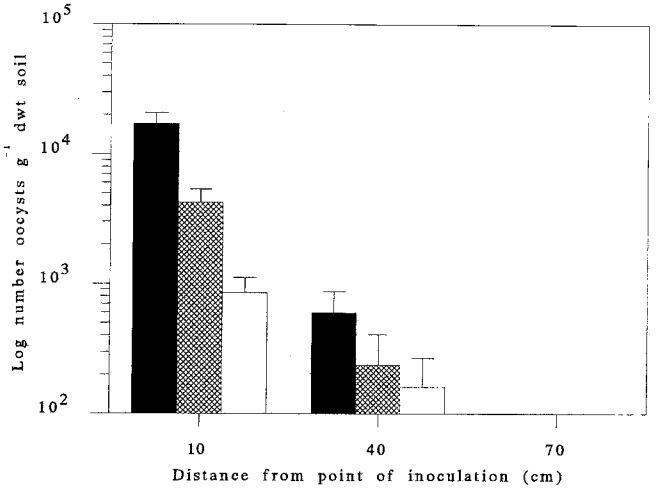

Fig. 5 Distribution of *Cryptosporidium parvum* oocysts remaining within the soil blocks at different depths down the soil profile at the end of the 70-day study (■ top 6 cm, ⊠ middle 7 cm, □ bottom 7 cm); values are the averages from the three replicate blocks. *Bars* represent standard errors

within the soil. The majority of oocysts were found in the upper 6 cm of soil at the top end of the block (Fig. 5), i.e. at the point nearest the application site, numbers decreasing as the depth down the soil profile increased. At 40 cm away from the line of inoculation numbers were again lower and in the middle and bottom 7-cm portions were at the limits of detection, with some replicates giving counts just above the detection limit and others being below quantifiable limits. No oocysts were detected at any depth in the soil at 70 cm (i.e. at the opposite end of the block to which the inoculated slurry had originally been applied).

Discussion

Although studies have demonstrated the role of runoff from both grazed and waste-treated land in decreasing water quality in terms of chemical or bacterial content (Doran and Lin 1979; Fernandez-Alvarez et al. 1991; Couillard and Li 1993), no published information is available on how protozoans such as *Cryptosporidium* may be transported by this route. Thus the aim of our study was to determine the extent to which *Cryptosporidium* oocysts applied to grassland in slurry would be transported in runoff following irrigation. The movement of the pathogen with water percolating down through the soil was also monitored, as was the location of oocysts remaining within the soil at the end of the 70-day study.

The tilting soil table system which we used to simulate movement of runoff across agricultural land proved a very successful model system which completely avoided the environmental problems associated with the application of *Cryptosporidium* to farmland. However, our initial trials to check the water flow across and down soil blocks following irrigation showed that in two of the three blocks the majority of water travelled down through the soil profile rather than across the soil surface. In the third block approximately half the added water travelled across the surface as runoff, the remainder being lost as leachate. These variations are probably explained by differences in soil composition and structure in different areas of the field from which the soil blocks were taken. Although the soil type selected was a poorly draining silty clay loam soil, variations in the depth and distribution of the heaviest clay fraction throughout the field could have resulted in the three blocks containing different proportions of clay and consequently having different permeabilities. Alternatively it is possible that the differences in permeability were due to differences in soil macropore structure caused, for example, by variations in root channels or earthworm populations. Earthworm populations may significantly affect the movement of microorganisms in soil either indirectly by preferential water flow along worm channels, or indirectly by transport with the worm, either on the surface of the worm (Thornton 1970) or following passage through the gut (Hendriksen 1995). In all three soil blocks less water was lost as runoff than had been expected, with more percolating down through the soil as leachate, despite the fact the soils had been thoroughly wetted. This may have been due to the uppermost layers of soil (as used in this study) being more permeable than the complete soil profile as measured in the field, where the soil frequently suffered from waterlogging. It should thus be borne in mind that the results for oocyst movement in runoff obtained in this study may be an underestimate of that which would be obtained on a truly impermeable heavy clay soil where greater volumes of water would be lost as runoff.

Block 2 continued to lose significantly more of the applied water as runoff (in comparison with leachate) than either of the other two blocks over the entire study. This was paralleled by higher numbers of oocysts being lost in the runoff from this block, with losses continuing over the

whole 70 days of the study. Patterns for the release of pathogens in runoff have also been found to be extended over long periods in other studies. Jawson et al. (1982) showed that faecal coliform counts in runoff exceeded water quality standards for up to a year after cattle were removed from land, similarly illustrating that problems from contaminated runoff may persist for long periods after the source of the problem has been removed. Even in blocks 1 and 3 where the volume of water lost as runoff was relatively small, oocysts could still be detected in runoff for as long as 4 weeks after application of the waste. This suggests that even small volumes of runoff may have serious consequences for contamination of water courses. Thus, even on land where the majority of water is lost by leaching down through the soil profile, the possibility of pollution from occasional runoff should be seriously considered. Similarly, the potential for pathogen transfer through percolation on land where the major problem is perceived to be runoff should be considered, as we detected significant numbers of oocysts in leachate percolating down through the soil over the entire period of study. A major factor influencing the seriousness of the problems associated with runoff is the survival characteristics of the pathogen in the environment (Walker et al. 1990). Obviously, if survival is limited problems associated with their subsequent transport are significantly reduced. Previous studies (Mawdsley 1994) have shown that significant numbers of oocysts are capable of surviving in soils for periods in excess of 60 days under conditions similar to those used in this study. Hence, the majority of oocysts being lost in both the runoff and leachate from the current study would probably have been viable and therefore a pollution threat.

Due to our limited ability to cope with the processing of large volumes of liquid, irrigation rates in this study were intentionally kept low. If parallels are drawn with other work done with bacterial pathogens (Patni et al. 1985) if irrigation rates were increased, the numbers of oocysts contained in runoff would be expected to rise. Similarly, based on the results of studies examining bacterial and chemical pollution in runoff, other factors such as different slopes, the distance between the pollutant and the water source and different times or methods of waste application (e.g. surface vs subsurface) would also be expected to affect the degree of rate of pollution (Khaleel et al. 1980; Culley and Phillips 1982; Baxter-Potter and Gilliland 1988; Couillard and Li 1993) and hence warrant further investigation.

At the end of the study soil samples were taken to establish how far oocysts had been transported within and across the soil block. At the top end of the block (i.e. nearest to the point of application) oocysts could be detected throughout the 20-cm profile in all three blocks although numbers decreased with increasing depth. Numbers of oocysts in the soil at 40 cm from the point of introduction were considerably lower, again decreasing as the soil profile was descended. In all three blocks numbers in the middle and bottom 7-cm portions were at the limits of detection, with a few samples giving counts but most being below quantifiable limits. At the furthest end from the point of inoculation, no oocysts could be detected in the soil at any depth. This suggests that oocysts transported in runoff are not adsorbed onto soil particles to any great extent as the runoff flows over the soil surface, and therefore are not later washed down into the soil profile. It seems more likely that oocysts will either be washed away in the runoff or leached down into the soil close to their point of introduction. When leaching down into the soil profile did occur, the results were similar to those seen in previous studies investigating the vertical transport of *C. parvum* oocysts through intact soil cores (Mawdsley et al. 1996). In those studies although oocysts were washed through 30-cm soil cores and detected in the leachate, large numbers were retained within the soil matrix, the majority being located in the top few centimetres, numbers subsequently decreasing with increasing depth.

This study has demonstrated the potential pollution threat that exists when contaminated livestock waste is applied to land. Following rainfall the transfer to the aqueous environment of pathogens contained in the waste may occur either in runoff across the soil surface or by leaching down through the soil profile, depending on the characteristics of the soil. Although the distances travelled in our controlled environment study are significantly less than would be needed to cause contamination in field situations, the fact that oocysts appeared to be retained within the runoff (and not lost by adsorption to soil as it ran over the soil surface) suggests that they would probably be transported over longer distances and hence may pose a significant pollution risk.

Acknowledgements This work was funded by a MAFF/SOAFD open contract 'Protozoan, bacterial and viral pathogens, farm animal wastes and water quality protection'. The authors would like to thank Mr. D. Mason for help with design and construction of the soil tilting table apparatus, and Dr. M.K. Theodorou for useful discussion of the manuscript.

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