Evidence for the existence of Cryptosporidium oocysts as single entities in surface runoff

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Abstract There is uncertainty whether Cryptosporidium oocysts attach to particles or to each other under ambient water conditions. Particle size distributions of Cryptosporidium oocyst suspensions were determined over a range of ionic strengths and pHs to determine under those environmental conditions that may promote oocyst aggregation. Cryptosporidium oocysts were shown to only aggregate in high ionic strength solutions (> 0.45 M) and remain largely as single entities at ionic strengths and pHs that were likely to be encountered in surface runoff. Similarly, in loam soil suspensions, rather than attaching to the soil particles the majority of oocysts also remained as single entities. Overall, oocysts are expected to remain largely unattached to either themselves or soil particles in overland runoff. This has implications for pathogen transport and modelling since oocysts that are freely suspended are more likely to be transported in runoff to surface waters than if attached to more dense soil/faecal particles.

Keywords Cryptosporidium aggregation

Introduction
Cryptosporidium parvum is a protozoan parasite that is ubiquitous in the environment and has a worldwide distribution (Packham, 1990). Cryptosporidium oocysts have been found in the surface waters of almost all continents (Horman et al., 2004; Kfir et al., 1995; Ionas et al., 1998; Lisle and Rose, 1995), and waterborne outbreaks of cryptosporidiosis have been reported since the 1980s. Oocysts find their way into surface waters through direct deposition by sewage effluent, direct animal faecal deposits, or wastewater discharges, while indirect deposition can result from overland transport in runoff from land affected by grazing domestic and wild animals or farming practices such as spreading of manure.

Knowledge of the way Cryptosporidium oocysts attach to particulate matter in the environment is essential information for modelling the movement of oocysts in overland runoff during rain events. Although there are numerous publications on the levels of Cryptosporidium oocysts in water bodies (Lisle and Rose, 1995; Bodley-Tickell et al., 2002) and several who have shown that transport occurs through soil (Mawdsley et al., 1995, 1996a,b; Brush et al., 1998), overland transport mechanisms from faecal pat to stream are not well understood (Dai 2003, Davies et al. 2004). Recent publications have suggested that Cryptosporidium oocysts have a low tendency to attach to particles or each other at neutral pH (Dai and Boll, 2003; Butkus et al., 2003). Information about the survival and movement of Cryptosporidium oocysts is required to model oocyst transport in drinking water catchments, for risk assessment, and to develop effective control practices such as vegetated riparian buffer zones (Atwill et al., 2002). Here we describe studies of ionic, pH and soil aggregation of oocyst suspensions.
Methods

Preparation of Cryptosporidium oocysts

Faecal samples were obtained from 7-21 day old calves at a farm in the outer Sydney suburb of Bringelly, NSW Australia. This farm has a dairy herd that is endemically infected with predominantly one strain of Cryptosporidium (Blasdall et al., 2002). Faecal smears were stained with a fluorescein isothiocyanate (FITC) labelled monoclonal antibody (EasyStain, BTF, Australia) and viewed by epifluorescence microscopy to determine which faecal slurries contained the highest numbers of Cryptosporidium oocysts. Oocysts were extracted from the faecal suspensions by diethyl ether defatting and sucrose flotation (Upton, 1997). Oocysts used in the aggregation study were then filtered through 11 µm pore size nylon netting (Millipore, Australia).

Aggregation study

Particle size distributions were determined using a Malvern Mastersizer E to measure aggregation of Cryptosporidium oocyst suspensions, with the formation of aggregates confirmed by epifluorescence microscopy. A low ionic strength solution (0.01M KNO$_3$) containing approximately $10^6$ oocysts was adjusted to pH values between 5.23 and 8.96 and the particle size distribution measured. Similarly, particle size distributions of oocyst suspensions were measured at pH 3.5 over a range of ionic strengths that were adjusted using MgCl$_2$.

Settling column study

Settling columns were prepared in 6 × 43 cm cylinders by adding artificial rainwater (4.07 mg NaNO$_3$, 3.24 mg NaCl, 0.35 mg KCl, 1.65 mg CaCl$_2$.2H$_2$O, 2.98 mg MgSO$_4$.7H$_2$O and 3.41 mg [NH$_4$]$_2$SO$_4$ per litre of ultra pure water) as per Laegdsmand et al. (1999) to 20 g of sieved loam soil to a final volume of 1 litre. Cryptosporidium oocysts (approximately $10^6$) were added and, after mixing and standing for one hour to allow any oocyst attachment to surface loam soil particulates, the suspensions were mixed thoroughly by inversion before particles were allowed to settle without being disturbed.

Different particle size fractions within the columns were collected by exploiting the size-dependant settling velocities of particles of varying sizes. The settling velocities were estimated using a modification of Stoke’s law ($V = kd^2$, where $V$ is the velocity of fall of the particles, $k$ is a constant combining density, gravity and viscosity, and $d$ is the diameter of the particles) (Palmer and Troeh, 1995). Actual particle size distribution and Cryptosporidium concentrations were measured in fractions collected 10 cm below the surface of the settling columns at regular intervals over 15 hours (900 minutes). Cryptosporidium oocysts were filtered onto membranes (0.8 µm pore size ATTP Isopore$^+$ membrane filters, Millipore, Australia) and stained with EasyStain. Oocysts were viewed using epifluorescence microscopy and counted.

Results and discussion

Aggregation study

The particle size profiles of Cryptosporidium oocyst suspensions in low ionic strength solutions between pH 3.30 and 9.96 are shown in Figure 1. A single peak was obtained in the particle size range 3.5–4.3 µm for each pH value tested, indicating that there was little tendency of the oocysts to aggregate over this pH range at low ionic strengths.

Figure 2 shows the particle size profiles at pH 3.5 in varying ionic strengths. The lower pH was used in an attempt to promote oocyst aggregation. The isoelectric point of oocysts is reported to be around pH 2 (Karaman et al., 1999). Using a low pH therefore
increases the probability of self aggregation due to decreasing the electrostatic repulsion between the oocysts. A single peak over a narrow size range was obtained in the particle size range 3.5–4.3 μm up to 0.45 M, indicating little tendency of oocysts to aggregate at the lower ionic strengths tested. Few oocysts remained as single entities above an ionic strength of 2.36 M.

**Settling column study**

Particle sizing of samples collected from settling columns at a depth of 10 cm at various times shows a settling of the heavier particles over time (Figure 3). The sampling times chosen related to the theoretical settling of particles of the size of single oocysts (4–6 μm). If there was little attachment to soil particles, then the number of oocysts at the sampling depth of 10 cm should remain constant until the 300 minute sampling. By the 300 minute sampling the 4–6 μm size oocysts should have settled more than 10 cm and theoretically would not be detected in the sampled portion. If the oocysts were attached to soil particles, then they would settle at a greater rate, and some losses prior to the 300 minute sampling would be expected.

**Figure 1** Particle size profiles of a *Cryptosporidium* oocyst suspension in 0.01 M KNO₃ at various pH values

**Figure 2** Particle size profiles of *Cryptosporidium* oocyst suspensions at various ionic strengths (pH 3.5)
The actual settling rates of the oocysts were compared to the theoretical rates by obtaining a particle size profile of the fractions collected at each time point. Although the peaks of the actual data at each sampling point were similar to that estimated using the modified Stoke’s Law, the spread of each peak showed that significant numbers of particles larger than that determined by the calculation were still present in each sample. It is possible, due to the large volume of sample that was removed for analysis at each time point, that some particulates that had settled below a depth of 10 cm may have been disturbed and collected during the sampling process.

Students-Newman-Keuls (SNK) analysis ranked the Cryptosporidium results (Table 1) into three groups, with groups A and B overlapping (Table 1). This indicates that there was no significant difference in oocyst concentration in the different fractions between 0 and 300 minutes. Although the measured particle size distributions show that the highest percentage by volume of total solids in the 300 minute fraction were particles in the size range 2–2.4 μm, the entire range of particle sizes present was up to 11.6 μm. Therefore, in the 300 min fraction there were still a large number of particles of a similar size to Cryptosporidium oocysts present. Similarly, although the 900 minute fraction showed a significantly lower number of Cryptosporidium, the entire range of the particles present in the fraction (0.5–6.3 μm) still contained a proportion of particles that were of a similar size to Cryptosporidium oocysts.

The overlapping SNK rankings A and B for the concentration of oocysts counted in the sampled fractions indicates that a low number of oocysts may have attached to some particles in the column and therefore settled faster than single oocysts. The fraction sampled at 88 minutes has a measured peak particle size similar to that obtained for oocyst suspensions (Figures 1 and 2). The oocyst count for this fraction is statistically ranked both with the 300 minute fraction (ranking B), where both single oocysts have been removed by settling, and the initial fraction (ranking A). This non-delineation of the rankings makes it difficult to estimate the low percentage of oocysts likely to be attached to soil particles. Approximately 20% of the oocysts present were removed with particles of the size range 64–123 μm. For sediment modelling, particles less than 64 μm in size are often grouped together and modelled as colloidal particles. However, whether or not...
Cryptosporidium oocysts aggregate together is important information for pathogen modelling. Dai (2003) suggested that Stoke's Law was inadequate for predicting oocyst settling times in columns since the Cryptosporidium oocyst settling rates decreased over time. Possible explanations for this phenomenon were increasing the density of the water in the lower parts of the column by the concentration of particles as they settled, or aggregation where a non-spherical aggregate could create a lot more drag as it moves through the water.

**Conclusions**
Given that the majority of oocysts mixed with a loam soil appeared to be freely suspended according to the particle size profiles and that the tendency for oocysts was to remain as single entities in pH and ionic strength conditions normally encountered in the environment, it is more likely that the Cryptosporidium are transported as single oocysts rather than attached to more dense soil particles. This has implications for pathogen transport and modelling since oocysts that are freely suspended are more likely to be transported in runoff to surface waters than if attached to more dense soil/faecal particles.

**Acknowledgements**
We acknowledge the financial support of the Cooperative Research Centre for Water Quality and Treatment and the American Water Works Association Research Foundation. Also, assistance with the use of Stoke’s law from Justin Brooks is gratefully acknowledged.

**References**


