REVIEW ARTICLE

Critical processes affecting *Cryptosporidium* oocyst survival in the environment

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SUMMARY

Cryptosporidium are parasitic protozoans that cause gastrointestinal disease and represent a significant risk to public health. *Cryptosporidium* oocysts are prevalent in surface waters as a result of human, livestock and native animal faecal contamination. The resistance of oocysts to the concentrations of chlorine and monochloramine used to disinfect potable water increases the risk of waterborne transmission via drinking water. In addition to being resistant to commonly used disinfectants, it is thought that oocysts can persist in the environment and be readily mobilized by precipitation events. This paper will review the critical processes involved in the inactivation or removal of oocysts in the terrestrial and aquatic environments and consider how these processes will respond in the context of climate change.

Key words: *Cryptosporidium*, survival, environment, inactivation, processes, review, climate change.

INTRODUCTION

Protozoan parasites of the genus *Cryptosporidium* are ubiquitous and a significant enteropathogen of a wide range of vertebrates, including mammals, birds, reptiles, and fish (O'Donoghue, 1995; Sturdee *et al*. 1999; Fayer *et al*. 2000*a*; Sreter and Varga, 2000; Alvarez-Pellitero and Sitja-Bobadilla, 2002; Xiao *et al*. 2004). They are the cause of the gastrointestinal disease cryptosporidiosis, which primarily involves watery diarrhoea in mammals and birds and gastritis in reptiles and fish (O'Donoghue, 1995). While the disease is normally self-limiting, persistent infections have been associated with severe, chronic disease, particularly in snakes or immuno-compromised mammals (Current *et al*. 1983). Recent research has identified drugs (e.g. nitrazoxanide) for the treatment of cryptosporidiosis, making this less of a concern in immunocompromised human patients (Smith and Corcoran, 2004; Rossignol *et al*. 2006).

The infectious form is the oocyst, and a single oocyst is sufficient to produce infection and disease in an animal model (Pereira *et al*. 2002). In humans, the median infectious dose for some isolates of *C. parvum* has been reported to be as low as 12 oocysts or as high as 2066 oocysts (Messner *et al*. 2001). Transmission can occur via the faecal/oral route or by ingestion of contaminated food or water, the latter of which serves as an excellent vehicle for its transmission (Juranek, 1997). Following ingestion by the host of an infectious oocyst, exposure to stomach acid, bile salts and host metabolic temperature promotes destabilization of the oocyst wall, resulting in release of the sporozoites that can then infect the epithelial cells lining the luminal surfaces of the digestive and respiratory tract of the host (O'Donoghue, 1995; Chen *et al*. 2004). The life-cycle is complex, comprising asexual and sexual stages, with the sexual cycle resulting in the production of millions of thick walled, environmentally robust oocysts (Fayer *et al*. 2000*a*; Atwill *et al*. 2003), which are excreted with the faeces of the host and subjected to the rigors of the environment until rendered non-infectious or ingested by a susceptible host (Fayer *et al*. 1998*a*).

Cryptosporidium sparked enormous public health interest after the large human waterborne outbreak in Milwaukee in 1993 (MacKenzie *et al*. 1995). In the past decade, *Cryptosporidium* has been identified as the cause of numerous outbreaks of waterborne disease affecting hundreds of thousands of individuals (SoloGabriele and Neumeister, 1996; Smith and Rose, 1998; Causer *et al*. 2006). However, because diagnosis of cryptosporidiosis is frequently not considered by many clinicians outside of the context of immunodeficient patients and many laboratories do not routinely test stool specimens for *Cryptosporidium* unless specifically requested, there

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is speculation that many cases of gastroenteritis caused by this parasite go undiagnosed and therefore unreported (Tillett *et al*. 1998; Hunter *et al*. 2001).

Cross-infection studies lead to the proposal that *Cryptosporidium* are zoonotic, with transmission reported between animals and humans (Meisel *et al*. 1976; Anderson *et al*. 1982; Reese *et al*. 1982). More recent genetic studies have demonstrated considerable genetic diversity among isolates of the same species suggesting that some species are in fact species complexes and that some of these species, such as *C. hominis*, may be host specific (Monis and Thompson, 2003). However, *Cryptosporidium parvum* is truly zoonotic and small percentages of human infections may also be caused by species other than *C. parvum* or *Cryptosporidium hominis* (Caccio, 2005). The widespread distribution of *Cryptosporidium* amongst vertebrates highlights the potential for transmission between host species (Sturdee *et al*. 1999; Hunter and Thompson, 2005).

Oocysts in the terrestrial environment are often associated with faeces from domestic and wild animals (Power *et al*. 2003) and can be readily mobilized by precipitation events (Davies *et al*. 2004). Consequently, oocysts of various species of *Cryptosporidium* are frequently detected in rivers and lakes and have also been detected in groundwater and treated drinking water (Smith and Rose, 1990; LeChevallier *et al*. 1995). In addition to being extremely resistant to chemical disinfection, oocysts can survive for several months in the aquatic environment (Robertson *et al*. 1992; Johnson *et al*. 1997). Therefore, *Cryptosporidium* represents not only a threat to public health but also a challenge to suppliers of drinking water.

The aim of this paper is to review the critical processes involved in the inactivation or removal of oocysts from both the terrestrial and aquatic environments, identify those processes that warrant further attention and consider how these processes will be impacted by climate change. A comprehensive understanding of these environmental matrices may help in mitigating the threat that *Cryptosporidium* oocysts pose by providing a valuable framework for risk assessment of *Cryptosporidium* oocysts in both the terrestrial and aquatic environments.

DETERMINATION OF *CRYPTOSPORIDIUM* OOCYST INACTIVATION

Studies investigating oocyst inactivation via biotic and abiotic mechanisms have used a variety of methods including animal infectivity or surrogate *in vitro* assays to determine oocyst viability. Before discussing critical processes affecting oocyst viability, it is prudent that the different methodologies used for determination of oocyst viability and the validity of these methods be considered.

A number of studies have examined oocyst survival using techniques such as *in vitro* excystation or vital dye staining (Robertson *et al*. 1992; Chauret *et al*. 1998), but such methods are only indictors of viability and are known to overestimate infectivity (Black *et al*. 1996; Bukhari *et al*. 2000). It therefore will be noted in this review where only these methods have been used for the determination of oocyst inactivation rates for particular biotic/abiotic stresses. Animal bioassays are considered the 'gold standard' for the assessment of *Cryptosporidium* oocyst infectivity, and the neonatal mouse model has been used extensively in the assessment of oocyst inactivation for *Cryptosporidium parvum* (Bukhari *et al*. 2000; Rochelle *et al*. 2002). However, this model is limited in its application for the assessment of *C. hominis* as this species cannot infect mice. While *C. hominis* ('human genotype') can be cultured in gnotobiotic pigs (Widmer *et al*. 1999), and this model has been used to assess drug efficacy (Theodos *et al*. 1998), it has not been used for the study of environmental oocyst inactivation.

Significant progress in the measurement of oocyst infectivity was catalysed by the development of cell culture (CC) assays for both *C. hominis* oocysts (Hijjawi *et al*. 2001) and *C. parvum* oocysts (Current and Haynes, 1984; Upton *et al*. 1994; Di Giovanni *et al*. 1999; Hijjawi *et al*. 2001; Rochelle *et al*. 2002). Evaluation of the cell culture assays using the human ileocecal adenocarcinoma (HCT-8) cells have shown them to be equivalent to the gold standard neonatal mouse infectivity assay (Shin *et al*. 2001; Rochelle *et al*. 2002; Slifko *et al*. 2002). A range of methods, including reverse transcriptase PCR (Rochelle *et al*. 1997), immunofluorescence microscopy (Slifko *et al*. 1997), colorimetric *in situ* hybridization (Rochelle *et al*. 2002) and real-time PCR (Keegan *et al*. 2003) have been applied for the analysis of CC infection. The combination of high throughput rapid molecular methods with CC, used to measure oocyst inactivation by temperature and solar inactivation (King *et al*. 2005, and manuscript in preparation), promises to improve our ability to rapidly quantify environmental oocyst inactivation through biotic and abiotic mechanisms. However, it is reasonable to consider that differences in reported inactivation rates for oocysts challenged by a particular stress may result from actual differences in the sensitivities of different methodologies employed by particular investigators to quantify oocyst inactivation. During this review we will endeavour to identify dissimilar methodologies used by investigators where significant differences in inactivation rates are reported for the same environmental stress.

TEMPERATURE

The ability of *Cryptosporidium* oocysts to initiate infection has been linked to finite carbohydrate

energy reserves in the form of amylopectin, which are consumed in direct response to ambient environmental temperatures (Fayer *et al*. 1998*b*). King *et al*. (2005), using ATP and CC-PCR assays, demonstrated that temperature inactivation at higher temperatures (up to 37 °C) is a function of increased metabolic activity. Temperature, therefore, is one of the most critical processes governing the fate of oocysts in the environment. While *Cryptosporidium* oocysts appear to be resilient to a wide range of temperatures (Chauret *et al*. 1998; Fayer *et al*. 1998*b*; Widmer *et al*. 1999; Freire-Santos *et al*. 1999, 2000*a*; Jenkins *et al*. 2000) increased holding temperatures correspond to decreased oocyst infectivity (Fayer *et al*. 1998*b*). At temperatures below 15 °C oocysts can maintain high levels of infectivity for periods of at least 24 weeks (Fayer *et al*. 1998*b*) with one report suggesting oocysts may remain infectious for periods longer than a year (Jenkins *et al*. 2002) (using 4′6-diaminidino-2-phenylindole (DAPI) and propidium iodide (PI) vital dyes). In contrast, at slightly increased environmental temperatures of 20 °C and 25 °C, inactivation is more rapid, leading to complete inactivation after 12 weeks and 8 weeks, respectively (King *et al*. 2005) (using CC-PCR). While Fayer *et al*. (1998*b*) (using neonatal BALB/c mice) described a longer survival time at these holding temperatures (24 weeks and 12 weeks for 20 °C and 25 °C respectively), they too described rapid inactivation rates as determined by reductions in mouse infectivity at these temperatures. At higher temperatures, King *et al*. (2005) described complete oocyst inactivation at 30 °C and 37 °C within 500 h and 72 h respectively. Therefore, *Cryptosporidium* oocysts, which have a finite energy supply, are highly susceptible to higher (>15 °C) environmental temperatures which they may encounter in the environment.

Oocysts are extremely susceptible to temperatures above 37 °C. Fayer (1994), using neonatal BALB/c mice, reported oocysts held at 64·2 °C for 5 min completely lost their ability to infect mice while Moriarty *et al*. (2005) reported that oocysts were rendered non-infective against monolayers of HCT-8 cells following treatments of 60 °C for 45 s and 75 °C for 20 s. Work in our laboratory (unpublished observations, using CC-PCR) has identified greater than 2 logs of inactivation at temperatures as low as 45 °C for holding times of only 20 min. These results suggest that oocysts are particularly susceptible to heat-shock. Oocysts may encounter such extreme temperatures, especially within manure, with bovine faecal material exhibiting temperature peaks over 40 °C and up to 70 °C if exposed to ambient temperatures of mid-20 to 30 °C under direct exposure to solar radiation (Li *et al*. 2005). Li *et al*. (2005) examined oocyst viability using a thermocycler to emulate the diurnal cycles found in bovine faeces and under such conditions they reported a complete loss of infectivity in the first diurnal cycle (using neonatal BALB/c mice). They determined that once climatic conditions generate internal faecal temperatures of greater than 40 °C, rapid inactivation occurs at rates of greater than 3 logs per day for *C. parvum* oocysts deposited in the faeces of beef and dairy cattle. Therefore, any substantial delays in time between the deposition of faeces containing *Cryptosporidium* and hydrological events such as rainfall, particularly under arid climatic conditions, will have the consequence that the initial load of infective oocysts may be significantly reduced by thermal inactivation.

The effects of low freezing temperatures also pose a serious challenge to oocyst survival. Fayer and Nerad (1996) reported that oocysts frozen at −10 °C for up to 168 h and then thawed out at room temperature were able to retain viability and infectivity to neonatal BALB/c mice. However, oocysts frozen at −20 °C for greater than 24 h and then thawed at room temperature did not infect mice. A similar study (using DAPI/PI vital dyes) by Robertson *et al*. (1992) found that after 21 h at −22 °C, 67% of oocysts were no longer viable. Kato *et al*. (2002) found that 99% of oocysts that were frozen at −10 °C in soils became inactivated within 50 days (using DAPI/PI vital dyes). Robertson and Gjerde (2004), using a vital dye assay, reported that oocysts did not persist in a Norwegian terrestrial environment over winter. They postulated that shear forces generated during the freeze-thaw cycles disintegrated the parasites. However, Fayer and Nerad (1996) predicted that for surface soil temperatures just below freezing and insulated by a cover of snow, oocysts may survive for weeks or months. The survival (measured using DAPI/PI vital dyes) of sentinel and control oocysts in field soil for 39 days (Jenkins *et al*. 1999*b*) adds weight to this prediction.

The predicted global temperature increases in the near future may have dramatic consequences for oocyst longevity in the environment, with small increases in temperatures above 15 °C increasing inactivation. However, warmer temperatures may also increase survival of oocysts in areas prone to soil subsurface freezing or lake ice covers, resulting in substantial numbers remaining infective after the winter period, where previously they may have been inactivated.

AMMONIA

Ammonia occurs naturally in the environment as a product of urea hydrolysis and of microbiological degradation of proteins and other nitrogen containing compounds (ammonification) (Jenkins *et al*. 1998). Other important sources of ammonia can include fertilizers, human and animal wastes, and by-products from industrial manufacturing processes

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(www.telwf.org/watertesting/ammonia.htm). Ammonia exists in 2 forms simultaneously, with the equilibrium between these forms governed largely by pH and temperature. These forms are NH_3 (un-ionized ammonia) and NH_4^+ (ionized ammonia or ammonium) and it is the NH_3 form that is particularly harmful to aquatic organisms (Arauzo and Valladolid, 2003). The formation of NH_3 is favoured at higher pHs but is also affected by increased temperature, so while the concentration of total ammonia may remain constant in a water body, the proportion of un-ionized ammonia fluctuates with temperature and pH. Significant formation of NH_3 can occur within a single day as water temperatures fluctuate (www.ec.gc.ca/substances/ese/eng/psap/final/ammonia.cfm).

Fayer *et al*. (1996) reported that oocysts suspended in water exposed to one atmosphere of pure ammonia at room temperature (21 °C to 23 °C) for 24 h were no longer infectious when fed to neonatal BALB/c mice, identifying ammonia as a useful disinfectant for oocysts. Jenkins *et al*. (1998) identified significant decreases in oocyst viability using DAPI/PI dye permeability and *in vitro* excystation assays. Based on their kinetic analysis, they predicted exposure to 0·06 M ammonia would inactivate 99·999% of freshly purified oocysts in 8·2 days at a temperature of 24 °C. The rate of inactivation for oocysts exposed to the same concentration at 4 °C was significantly less, with a hypothetical 55·1 days to reach 99·999% inactivation (Jenkins *et al*. 1999*a*). They concluded that environmentally relevant concentrations of free ammonia may significantly increase the inactivation of oocysts in ammonia-containing environments.

Significant concentrations of ammonia can be present in decomposing manure, especially manure storages (Muck and Steenhuis, 1982; Muck and Richards, 1983; Patni and Jui, 1991). Concentrations in cattle slurries have been measured at an initial concentration of 0·05 M, rising to 0·2 M after 3 weeks (Whitehead and Raistrick, 1993). According to ammonia induced oocyst inactivation data produced by Jenkins *et al*. (1998), exposures to such high concentrations would have significant effects on oocyst viability even at cool temperatures given longer exposure times (Ruxton, 1995). Therefore, storage of animal waste products may be regarded as an effective strategy to reduce oocyst numbers in livestock wastes before being spread onto the land (Hutchison *et al*. 2005).

While ammonia levels in manure storages may be high enough to substantially affect oocyst viability, Brookes *et al*. (2004) concluded that the impact of free ammonia on oocyst viability would be negligible in drinking water reservoirs. They calculated that levels of ammonium in the hypolimnion of lakes (often the highest concentration) are typically less than 1 mg/l, values considerably less than the lowest concentration tested by Jenkins *et al*. (1998), 0·007 M being equivalent to 1780 mg/l at a pH of 8 in an aquatic environment. Even for lakes undergoing eutrophication, ammonia levels according to these data would be too low to significantly affect oocyst viability. However, anthropogenic induced increases in ammonia levels in aquatic systems may have indirect effects on the survival of oocysts in aquatic systems through the disruption of benthic fauna and flora responsible for oocyst removal (see section on predation). Such fauna and flora may be significantly more susceptible to the lower ammonia levels that oocysts resist. Additionally, small increases in global temperature due to climatic change and the concomitant increase in water temperatures may raise ammonia levels in some water bodies. Such changes in the aquatic environment could possibly see an increase in the presence of oocysts due to slight increases in ammonia, to which some predatory organisms may be more susceptible.

Oocysts may reside in the soil for a considerable degree of time before mobilization by a precipitation event. Soils typically contain ammonia levels ranging between 1 and 5 ppm, which are not high enough to affect oocyst viability. However, freshly fertilized soils may contain levels as high as 3000 ppm (http://www.npi.gov.au/database/substance-info/profiles/8.html#env-whateffect), which is high enough to have an effect. It may be prudent to revisit the effect of ammonia on oocyst viability, but measuring inactivation using cell culture models instead of vital dye and *in vitro* excystation methods, which are too conservative for estimating reductions in oocyst infectivity. Any dramatic increases recorded in oocyst sensitivity to ammonia levels would have consequences for the predicted survival of oocysts, especially in manures, soils and possibly heavily polluted waters.

DESICCATION

While the robust nature of *Cryptosporidium* oocysts is well recognized and they are known to be resistant to many forms of environmental stress, desiccation is apparently an exception. Robertson *et al*. (1992) reported desiccation to be lethal with only 3% of oocysts viable as judged by DAPI/PI vital dye staining after being air-dried for 2 h at room temperature. Deng and Cliver, (1999), using PI vital dye staining, reported similar rates of survival with only 5% of oocysts remaining viable after 4 h of air drying at room temperature. This is in contrast to another coccidian, *Eimeria*, which has been reported to maintain viability under conditions of severe desiccation (Thomas *et al*. 1995).

Once oocysts are excreted into the terrestrial environment and released from faeces by precipitation or other physical processes, their survival above the soil milieu is greatly limited due to the process of

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desiccation. It is envisaged that this may vary greatly depending on the climatic setting, with increased rates of inactivation expected in more arid environments. However, once within the soil environment oocysts appear to be protected from desiccation as indicated by a majority of studies identifying soil moisture and soil water potential to have little effect on oocyst viability (see section on the physical terrestrial environment). Therefore, while those oocysts above the soil matrix may be extremely vulnerable to desiccation, those within it may be protected.

Since only vital dye assays have been used to measure oocyst inactivation from desiccation, it would be prudent to conduct further studies using the neonatal mouse or cell culture assays for more accurate measurements of inactivation. In addition, due to the limited number of studies undertaken it would be warranted to further investigate the process of desiccation by challenging oocysts to a variety of environmental conditions, with particular attention to synergistic interactions with temperature. In the light that climate change is predicted to increase the frequency and duration of droughts, it is possible that the process of desiccation may predominate in such areas as one of the critical processes inactivating oocysts.

THE PHYSICAL TERRESTRIAL ENVIRONMENT (SOIL MATRIX AND VEGETATION)

Once oocysts are shed in faeces, they may be released from the faecal matrix by the action of rainfall (Davies *et al*. 2004). After dispersal from the faecal matrix, inactivation may be dependent on the physical, chemical and biological properties of the soil environment (Ferguson *et al*. 2003). Jenkins *et al*. (2002) used DAPI/PI dye permeability and Davies *et al*. (2005) used fluorescent *in situ* hybridization (FISH) to estimate oocyst viability and found significant differences in oocyst survival in different soil types, identifying soil texture as important for survival. However, neither gave detailed explanations of how this parameter was able to influence oocyst viability, with the exception of Jenkins *et al*. (2002),who indicated that while unlikely, lower soil pH may contribute somewhat to this inactivation. Soil moisture in the ranges tested were not shown to be influential to oocyst survival (Jenkins *et al*. 2002; Kato *et al*. 2004; Davies *et al*. 2005), with the exception of a study by Nasser *et al*. (2003) that suggested oocysts in a loam soil can become susceptible to dehydration. Increased water potential (measured using osmotic potential as a surrogate for total water potential in soils, which can avoid problems such as heterogeneity in soil moisture distribution) has been identified as leading to oocyst population degradation (measured using PI vital dye and microscopy) (Walker *et al*. 2001). Therefore, it is possible that environmental soil moisture content may affect oocyst survival, but this requires further research effort for this to be conclusively determined. From the limited studies, biotic status appears to have little effect on oocyst inactivation within the soil environment (Davies *et al*. 2005). However, temperature (see above section) was identified (Jenkins *et al*. 2002; Davies *et al*. 2005) as the critical factor affecting oocyst survival within the soil profile, indicating that oocysts within the soil profile at 4 °C may remain infectious for very long periods (even years) regardless of soil texture.

Pathogen fate within the soil environment is not only a function of survival within the soil but also retention by soil particles (Zyman and Sorber, 1988). Soils have been shown to act as an effective pathogen filter, with a number of studies indicating that the majority of bacteria and/or viruses are removed in a relatively short distance (Cilimburg *et al*. 2000). Soil pore size may significantly affect the movement of protozoa through soil and protozoan cysts and oocysts are likely to be dependent on macropores for their transport and may be expected to show an even greater response to a lack of macropores in disturbed soils from precipitation events (Mawdsley *et al*. 1995; Davies *et al*. 2004). However, a study of the movement of *Cryptosporidium parvum* through 3 contrasting soil types identified distribution within the cores as similar in all 3 soil types, with the majority of oocysts in the top 2 cm of soil, and oocyst numbers decreasing with increasing depth (Mawdsley *et al*. 1996). Depending on soil saturation and soil type, this suggests the possibility for remobilization of those oocysts close to the soil surface with further precipitation events. Soils that consist predominantly of clay-sized particles can cake with wetting and drying, or freezing and thawing, and may pool water, establish water tables, and encourage runoff (Fuller and Warrick, 1985). However, Zyman and Sorber (1988) found that in soils with high clay content, adsorption plays an important role in virus removal. So it is possible that for less than saturating conditions, soils with higher clay contents may retain oocysts more readily under such conditions. Soil pH may also affect properties like adsorption (Mawdsley *et al*. 1996). However, Davies *et al*. (2005) identified little adsorption of oocysts in intact soils plots without vegetation.

Vegetation surfaces have been identified as very effective in reducing *Cryptosporidium* in surface runoff into drinking and irrigation water supplies (Tate *et al*. 2004; Trask *et al*. 2004). Grassland buffers of only 1·1 to 2·1 m in width have been shown to generate between 3 and 8 log retention of *Cryptosporidium* oocysts (Atwill *et al*. 2006), and in combination with soil type, vegetated buffered strips constructed on soils of lower bulk densities have been identified as most effective in retaining oocysts

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(Atwill *et al*. 2002). Davies *et al*. (2004), using intact soil blocks, showed that runoff load was significantly affected by vegetation status, the slope of the soil, and event characteristics in terms of rainfall intensity and duration. Based on their observations, a significant risk existed for the dispersion of oocysts from recent animal faecal deposits and their transport into nearby surface waters on sloping land of 10° or more with little or no vegetation after a short burst of rainfall of significant intensity.

On the soil surface, high temperature, desiccation and ultraviolet radiation (see relevant individual sections) can be lethal to pathogens. Therefore, oocysts within the soil column are to a large extent protected from inactivation depending on soil temperature and to a lesser extent soil texture. Oocysts within the soil column are tied away from host ingestion until a precipitation event can mobilize them. Climate change models predict more intense precipitation events in the future for a number of geographical locations (Easterling *et al*. 2000). Such scenarios may increase saturation of soil profiles, mobilize infectious oocysts within the soil column more often, and in combination with urbanization and deforestation of the landscape, may significantly increase the risk that *Cryptosporidium* oocysts pose. Further attention to particular watersheds at risk of oocyst contamination may therefore be warranted, so as to better predict source water quality.

SOLAR INACTIVATION

It is well established that solar radiation is a genotoxic agent with short-wavelength UV radiation, UV-B (280 nm to 320 nm) and UV-A (320 nm–400 nm), the most biologically damaging and mutagenic component of the electromagnetic spectrum (Caldwell, 1971; Ravanat *et al*. 2001). Although short-wavelength UV radiation can disturb most macromolecules, including proteins, lipids and nucleic acids, studies in animal systems suggest that damage to the structure and function of DNA is the primary mechanism responsible for cell injury and loss of viability by UV radiation (Friedberg *et al*. 1995; Malloy *et al*. 1997). UV exposure has been identified as being detrimental to a wide range of organisms including bacteria (Slieman and Nicholson, 2000; Whitman *et al*. 2004), fungi (Hughes *et al*. 2003), plants (Deckmyn and Impens, 1999; Ries *et al*. 2000; Hollosy, 2002) and animals (Misra *et al*. 2005). As well as impacting organisms on the terrestrial environment, surface irradiances are high enough to cause injurious effects in aquatic organisms even in coastal waters characterized by strong attenuation of UV radiation (Piazena and Hader, 1994). Therefore, oocysts in both terrestrial and aquatic environments are targets for solar inactivation.

While the efficacy of UV-C on *Cryptosporidium parvum* oocyst infectivity has been well documented during the last 10 years (for a review see Rochelle *et al*. (2005)), the effects of solar radiation on *Cryptosporidium* are little known. The effect of solar inactivation of *Cryptosporidium* has been limited to one study carried out in marine waters which identified a 90% reduction in viability (measured using excystation) after a 3-day exposure period (Johnson *et al*. 1997). Recent work has investigated the inactivation of *C. parvum* oocysts incubated in tap water and a range of environmental waters exposed to solar radiation over consecutive winter and summer periods (CC-PCR) (King *et al*., manuscript in preparation). These experiments, conducted on days with varying levels of solar insolation, identified rapid inactivation of *Cryptosporidium* oocysts in tap water, with up to 90% inactivation occurring within the first hour on the highest UV index days.

Results from these tap water inactivation experiments indicate that *C. parvum* oocysts are particularly susceptible to inactivation via solar insolation, indicating the potential for solar insolation to play a significant role in inactivating oocysts in the terrestrial environment. *Cryptosporidium* oocysts present on the soil surface may be exposed to the microbicidal effects of solar radiation and become quickly inactivated. While it is assumed that the majority of oocysts will be protected in the bulk of the soil matrix, including those in the top few centimetres of the bulk soil (Mawdsley *et al*. 1996; McGechan, 2002), precipitation events may re-mobilize oocysts and deposit them on top of the soil matrix, exposing previously protected oocysts to inactivation via solar radiation. This cycle may be repeated multiple times depending on the frequency of precipitation and presence of vegetation buffer zones which reduce oocyst runoff, therefore effecting significant reductions in the infectivity of the total oocyst load of the terrestrial environment.

Outdoor tank experiments have also identified rapid oocyst inactivation in environmental waters of varying water quality with up to 2 log inactivation recorded on a winter's day and up to 3 log inactivation recorded on a summer's day for *C. parvum* (King *et al*., manuscript in preparation). Dissolved Organic Carbon (DOC) content of the environmental waters was identified as significantly affecting oocyst induced solar inactivation, with increased DOC levels rapidly reducing oocyst inactivation. It is well known that in freshwater environments, the penetration of UV is dependent on the concentration and type of DOC as it is highly absorptive in the ultraviolet spectrum and determines the extinction co-efficient of UV light in a particular water body (Morris *et al*. 1995; Jerome and Bukata, 1998; Hutchison *et al*. 2005). Therefore, waters high in DOC can provide a natural shield to harmful solar radiation (Jerome and Bukata, 1998). However, while

solar inactivation of *Cryptosporidium* oocysts may be negligible in waters of high DOC content at depth, it may still play an important contributing factor to oocyst inactivation when oocysts mobilized by precipitation events are carried into such water bodies by warm water inflows. Inflows are controlled by their density relative to that of the lake, such that warm inflows will flow over the surface of the lake as a buoyant surface flow and cold, dense inflows will sink beneath the lake water where they will flow along the lake bed towards the deepest point (Brookes *et al*. 2004). With >90% oocyst inactivation occurring in just a few hours in the top 5 cm of environmental waters high in DOC (King *et al*., manuscript in preparation), oocysts present in warm water inflows into water bodies with strong UV light attenuation may still be strongly inactivated by UV light as they would be in the top few cm of the water column. Oocysts in water bodies with low DOC levels may, on the other hand, be quite vulnerable to solar inactivation at significant depths because UV can penetrate to a depth of 46 m in fresh water bodies (Brookes *et al*. 2004). However, those oocysts carried into water bodies by cold water inflows may escape the damaging effects of solar radiation if water bodies are high in DOC content.

Long-pass filter experiments have identified UV-B as the most germicidal wavelength (King *et al*. 2006). However, a pronounced but lesser effect on oocyst infectivity from UV-A (<380 nm) was also identified by the end of each experimental period. This is consistent with other findings that UV-A light also exhibits cytotoxic and mutagenic effects, however, to a smaller extent than UV-B (Ravanat *et al*. 2001). While UV-A may be less cytotoxic, it may have greater ecological significance, especially where oocysts are found in water bodies where lower wavelengths are more rapidly attenuated in the water column.

While it has been recently demonstrated that solar UV can substantially affect *Cryptosporidium* oocyst infectivity in environmental waters of varying water quality, suggesting that it may be a major factor driving oocyst inactivation in both terrestrial and aquatic environments, there is an enormous lack of data on solar inactivation rates in different environments. Previously, models for determining oocyst fate have incorporated solar radiation inactivation rates of other surrogate organisms (Brookes *et al*. 2004). However, data produced by King *et al*. (manuscript in preparation) suggests a greater degree of susceptibility to solar radiation than previously thought, warranting further effort to study the impact that solar radiation has on oocyst survival. For some environments, this may be the critical process determining oocyst inactivation.

Drastic stratospheric ozone depletion over the Antarctic and Artic, as well as moderate decreases in total ozone column over high and mid-latitude waters, have been reported (Hader *et al*. 1998). Changes in the spectral composition exceeding those experienced during the evolution of exposed organisms may pose significant stress for diverse aquatic ecosystems (IASC, 1995). Any anthropogenic increases in UV-B radiation from atmospheric ozone destruction may not just affect exposed oocysts in the terrestrial environment or upper water column of low DOC water bodies, but may affect oocysts deeper in the water column in higher DOC water bodies due increased solar UV photolytic degradation of dissolved organic carbon (Naganuma *et al*. 1996), resulting in a significant effect on oocyst survival in environments susceptible to such changes.

BIOTIC ANTAGONISM

It has been proposed that natural biological antagonism has a pronounced influence in determining the environmental stability of *Cryptosporidium* oocysts (Chauret *et al*. 1998). Yet to date, little work has been published on the predation of *Cryptosporidium* oocysts in either the terrestrial (Huamanchay *et al*. 2004) or aquatic environments (Fayer *et al*. 2000*b*; Stott *et al*. 2001, 2003), while no work has identified bacterial antagonism of oocysts. Rotifers, ciliates, amoebae, gastrotrichs and platyhelminths have previously been reported as capable of ingesting oocysts (Fayer *et al*. 2000*b*; Harvey, 2004; Huamanchay *et al*. 2004; Stott *et al*. 2001, 2003). However, there is minimal information on the effect that predation has on either the removal of oocysts from the environment, or on oocyst infectivity.

Fayer *et al*. (2000*b*) noted that rotifers excreted oocysts in boluses. King *et al*. (2005) also identified oocyst clumping in a number of raw water experiments, which was absent in the sterilized water controls, and concluded this to be a result of predation. It is therefore possible that oocyst predation and then excretion of oocysts in boluses or clumps may hasten the settling of oocysts in water bodies, removing them more quickly to the sediment (Brookes *et al*. 2004) (see Hydrological Parameters section). Brookes *et al*. (2004) noted that the feeding experiments reported in the literature exposed predatory organisms to prey densities greater than 10^4 oocysts/ml. This density is far greater than the oocyst density in the environment, which in a water reservoir can typically be 0 to 100 oocysts per 100 litres, leading to their suggestion that this represents an extremely low prey density for grazing (Brookes *et al*. 2004). However, of those organisms shown to ingest oocysts, many are phagotrophic size-selective filter feeders, therefore their prey range may include a plethora of particles in the same size range as *Cryptosporidium* oocysts. Prey and predatory densities may therefore be high enough to effectively ingest large numbers of oocysts finding their way into the aquatic environment. The effects of predation in

the natural terrestrial environment are unknown, with the limited oocyst microcosm studies in the soil environment not yet identifying any effect of microbial activity on oocyst survival (Davies *et al*. 2005).

Caenorhabditis elegans has been shown to ingest oocysts and excrete both intact oocysts and empty oocysts (Huamanchay *et al*. 2004). King *et al*. (2005) observed variation in oocyst FITC staining which they concluded could be a result of partial digestion of the oocyst wall due to predation of oocysts in raw water samples. Harvey (2004) found that DAPI- and FITC-stained oocysts were being degraded through the gut of a number of predatory organisms in feeding experiments. While empty oocysts can be safely assumed to be no longer infectious, it is not known if intact excreted oocysts are still infectious, or if these oocysts are as environmentally robust as they were before ingestion. It is also not known whether ingestion of oocysts by any of these organisms may act to protect oocysts from other biotic or abiotic stresses, potentially enhancing oocyst survival in the environment before ingestion by a susceptible host.

A number of RNA viruses have been identified as infecting protozoan parasites including *Leishmania* (Patterson, 1993), *Giardia* (Wang and Wang, 1986; Tai *et al*. 1996), *Trichomonas*, *Eimeria* and *Babesia* (Wang and Wang, 1991). Viral-like double-stranded RNAs and virus-like particles have also recently been identified in *Cryptosporidium* sporozoites (Khramtsov and Upton, 1998, 2000). However, it is unknown whether the presence of the virus affects sporozoite infectivity, survival, or fitness. It is therefore interesting to speculate that oocysts containing infected sporozoites may be more susceptible to environmental degradation through synergistic interactions with other stresses. For example, increased metabolic temperatures may result in increased viral replication within the sporozoite, increasing metabolic demand, therefore reducing the longevity of the oocyst in the environment.

Invertebrates, such as dung beetles which feed on dung, can rapidly degrade manure pats and reduce the activity of other organisms within the pat such as flies, fungi and nematodes (Fincher, 1975; Beesley, 1982; Biggane and Gormally, 1994). Such activity may also reduce the survival of *Cryptosporidium* oocysts in faeces either directly by feeding activity and ingestion and inactivation of oocysts (Mathison and Ditrich, 1999) or through the breakdown of the dung pat and exposure to abiotic stresses such as solar radiation and desiccation. Considering the enormous oocyst load in animal faeces, the activity of dung beetles may significantly impact on the survival of oocysts in the terrestrial environment, and the study of this warrants further effort. However, invertebrates have also been implicated in the spread and dissemination of *Cryptosporidium* oocysts (Graczyk *et al*. 2000, 2005; Follet-Dumoulin *et al*. 2001; Szostakowska *et al*. 2004). The feeding mechanisms, breeding habits and indiscriminate travel between filth and food make some groups of insects, such as non-biting flies and cockroaches, efficient vectors of protozoan parasites of concern to human health (Graczyk *et al*. 2005).

Many potential predators of *Cryptosporidium* oocysts may be found in animal faeces, the soil or aquatic environments. Invertebrate organisms may pose both negative and positive stresses on oocyst survival. Further experiments designed to measure oocyst removal or attenuation from the environment due to such biotic processes are needed if we are able to effectively model the fate of *Cryptosporidium* oocysts in the environment. Until we know which organisms are responsible for removal, attenuation, dissemination or reduction in general fitness of oocysts, it is not possible to predict the effect that climate change may have on these organisms and therefore the fate of oocysts in the environment.

HYDROLOGICAL PARAMETERS

Once oocysts escape the terrestrial environment and enter the aquatic ecosystem, water can serve as an excellent vehicle for their transmission and subsequent contact with and ingestion by hosts. However, there are a number of important processes controlling the transport and distribution of pathogens within water bodies. These include dispersion, dilution, horizontal and vertical transport. The settling of pathogen particles and their partition into the sediment (hydrodynamic processes) is discussed in detail by Brookes *et al*. (2004).

Horizontal transport is predominantly driven by inflows and basin-scale circulation patterns including wind-driven currents and internal waves. The riverine inflow is considered to be a major source of pathogens to water bodies. Inflows are controlled by their density relative to that of the lake, such that warm inflows flow over the surface of the lake and cold dense inflows sink beneath the lake water. As already discussed, this can impact enormously on the solar radiation exposure oocysts receive. The inflow will entrain water from the lake, increasing its volume and diluting the concentration of oocysts. The speed at which an inflow travels through the lake, its entrainment of lake water and resulting dilution of its characteristics and its insertion depth are all of critical importance in determining the hydrodynamic distribution of oocysts (Brookes *et al*. 2004).

The vertical distribution of pathogens can be affected by the settling rate of the pathogen, which in turn is affected by its size and density (Reynolds, 1984). Aggregation of pathogens to particulate material or integration into an organic matrix will influence settling. Medema *et al*. (1998) identified

individual oocysts with a settling velocity in water of 0·03 m/day, and when attached to particles from biological effluent the rate increased to 2·5 m/day. Hawkins *et al*. (2000) estimated sedimentation rates of oocysts of 5–10 m/day. Therefore, while the settling of individual oocysts is extremely slow, when attached to other particles this can increase their settling velocity by 2 orders of magnitude (Brookes *et al*. 2004). Therefore, the size of particles with which *Cryptosporidium* associates is a major factor influencing its transport in a water body. However, a study by Dai and Boll (2003) determined that oocysts do not attach to natural soil particles and would travel freely in water. This is supported by the negative surface charge of oocysts at neutral pH, suggesting that they would not readily adsorb to particles (Ongerth and Pecoraro, 1996). However, this conflicts with the high settling velocities recorded by Hawkins *et al*. (2000) and Medema *et al*. (1998), suggesting that in some situations oocysts must associate with larger particles. Brookes *et al*. (2004) suggested that the aggregation of oocysts to particles in water will be primarily controlled by turbulence, therefore if aggregation is to occur it is much more likely in inflowing rivers than within lakes and reservoirs due to the higher rates of turbulence in riverine systems. Vertical transport may also be affected by internal waves, which may generate significant vertical movements in the order of tens of metres, resulting in the vertical advection of pathogens and particles (Deen and Antenucci, 2000; Brookes *et al*. 2004).

While sedimentation of oocysts may remove them from host ingestion, it may only be temporarily. Since oocysts can remain viable for lengthy periods of time within the sediment, especially if cold and dark, any re-suspension and subsequent redistribution will be important in estimating the risk such oocysts still pose. Turbulence generated by underflow events and internal waves can result in sediment re-suspension of particulate material (Michallet and Ivey, 1999). If the turbulent zone of benthic boundary layers coincides with the zone of substantial sediment accretion, then large amounts of suspended oocysts may occur in this region. Climate change predictions forecast more intense precipitation events, this may in turn result in increased disturbance of sediments and re-suspension of infectious oocysts; however it may also result in increased settling of oocysts due to increased turbulence combined with increased organic matter in waters in which oocysts may become enmeshed.

SALINITY AND ACCUMULATION IN FILTER FEEDING SHELLFISH

Large quantities of oocysts find their way to the ocean from precipitation events or through the discharge of treated and untreated waste products, resulting in contamination of marine waters. Any survival of oocysts for significant periods at environmental temperatures provides potential for exposure to humans and marine animals. Significant reductions in oocyst viability have been identified in seawater trials using DAPI/PI vital dyes (Robertson *et al*. 1992), with concentrations of 20 ppt and higher demonstrated to have a significant effect on *Cryptosporidium* infectivity (Fayer *et al*. 1998*a*) (neonatal BALB/c mice). Salinity, time and salinity-time interactions have been described as important factors affecting infection intensity (Freire-Santos *et al*. 1999) (neonatal CD-1 mice). Fayer *et al*. (1998*a*) also identified a strong synergistic interaction of salinity and temperature, with oocysts held at 20 °C infectious at salinities of 0 and 10 ppt for 12 weeks, 20 ppt for 4 weeks, and 30 ppt for 2 weeks. While these findings demonstrate that salinity can have a pronounced effect on oocyst viability, they also suggest that oocysts could survive in marine waters long enough to be removed by filter feeders or infect marine animals. This is supported by the identification of *Cryptosporidium* species in marine mammal species (Fayer *et al*. 2004; Appelbee *et al*. 2005; Hughes-Hanks *et al*. 2005) fish (Sitja-Bobadilla *et al*. 2005) and the detection and recovery of infectious oocysts in filter feeding shellfish worldwide (Fayer *et al*. 1998*a*, 2002; Freire-Santos *et al*. 2001, 2000*b*; Gomez-Couso *et al*. 2003, 2004; Giangaspero *et al*. 2005; MacRae *et al*. 2005). Because shellfish are able to filter large volumes of water and concentrate oocysts, this poses a threat not only to human health but to marine wildlife that may feed on these shellfish as well. With increased global temperatures predicted and subsequent estimates of large rises in sea levels due to melting of the Artic and Antarctic ice sheets (Overpeck *et al*. 2006), any large decreases in salinity or ocean freshening (Wadhams and Munk, 2004) may result in increased survival of oocysts. Any lengthening of the period of exposure for marine wildlife to oocysts may have detrimental consequences for marine ecosystems due to increased parasitism.

CONCLUSIONS

While *Cryptosporidium* oocysts are considered to be environmentally robust, they are sensitive to a number of abiotic and biotic processes that they may encounter in either the terrestrial or aquatic environment. While a number of these processes (e.g. temperature) have been well quantified by researchers, other processes affecting oocyst viability (e.g. solar radiation/biotic antagonism) need much more attention. Importantly, it is largely unknown what synergetic processes occur between these different stresses and how they affect oocyst survival and/or viability in the environment. When further studies are undertaken, attention must be paid to

the methodology used to measure oocyst inactivation. Using either the neonatal mouse assay or cell culture assays for measuring oocyst viability (instead of vital dye or excystation) after being challenged by these stresses will help provide accurate data for estimating *Cryptosporidium* risks in different environments.

While much progress has been made in the disinfection of oocysts in treated water supplies using artificial UV-C (Clancy *et al*. 2004; Johnson *et al*. 2005; Hijnen *et al*. 2006), it is important to realize that the vast amount of potable water used for consumption by the world's populus will not be disinfected using such processes and *Cryptosporidium* oocysts will continue to pose a threat to many communities, as well as impacting wildlife and domestic animals. Climate change and climate warming have been predicted to increase pathogen development and survival rates, disease transmission and host susceptibility. However, while the severity and frequency of diseases are predicted to increase for many host-parasite systems, a subset of pathogens might decline, releasing hosts from disease (Harvell *et al*. 2002). Our analysis of the critical processes involved in the inactivation and removal of oocysts from the environment leads us to predict that while some regions of the world will experience increasing incidences of cryptosporidiosis, other areas will see a decline in the disease. Further attention to those critical processes affecting oocyst survival in particular environments will help us to determine which areas may become more susceptible to outbreaks of cryptosporidiosis. Finally it has not escaped our attention that the processes discussed in this review and how they may respond to climate change will also have important implications for other coccidians with an infectious oocyst stage. Any substantial changes in the levels of host parasitism by coccidian parasites will have important ramifications for the ecology of those particular systems.

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