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Review

Use of aerobic spores as a surrogate for cryptosporidium oocysts in drinking water supplies

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ABSTRACT

Waterborne illnesses are a growing concern among health and regulatory agencies worldwide. The United States Environmental Protection Agency has established several rules to combat the contamination of water supplies by cryptosporidium oocysts, however, the detection and study of cryptosporidium oocysts is hampered by methodological and financial constraints. As a result, numerous surrogates for cryptosporidium oocysts have been proposed by the scientific community and efforts are underway to evaluate many of the proposed surrogates. The purpose of this review is to evaluate the suitability of aerobic bacterial spores to serve as a surrogate for cryptosporidium oocysts in identifying contaminated drinking waters. To accomplish this we present a comparison of the biology and life cycles of aerobic spores and oocysts and compare their physical properties. An analysis of their surface properties is presented along with a review of the literature in regards to the transport, survival, and prevalence of aerobic spores and oocysts in the saturated subsurface environment. Aerobic spores and oocysts share many commonalities with regard to biology and survivability, and the environmental prevalence and ease of detection make aerobic spores a promising surrogate for cryptosporidium oocysts in surface and groundwater. However, the long-term transport and release of aerobic spores still needs to be further studied, and compared with available oocyst information. In addition, the surface properties and environmental interactions of spores are known to be highly dependent on the spore taxa and purification procedures, and additional research is needed to address these issues in the context of transport.

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1. Introduction

Waterborne diseases pose a significant threat to public health worldwide. Approximately 1.8 billion people utilize a water source that is contaminated by feces (Bain et al., 2014) and waterborne diseases kill about 2.1 million people worldwide every year (Pruss et al., 2002). In the United States, the Environmental Protection Agency (EPA) has continually modified its rules for the monitoring and treating of public water systems to adapt to changes in understanding of how pathogens are transported in surface water and groundwater. The Long Term 2 Enhanced Surface Water Treatment Rule (LT2 rule) was established in 2006 to reduce the risk of microbial contamination in drinking water by targeting public water sources for increased monitoring and treatment if the source is a surface water or groundwater under the direct influence (GWUDI) of surface water (U.S. EPA, 2010). The EPA defines GWUDI as "any water beneath the surface of the ground with significant occurrence of insects or other macroorganisms, algae, or large-diameter pathogens such as *Giardia lamblia* or *Cryptosporidium*, or significant and relatively rapid shifts in water characteristics such as turbidity, temperature, conductivity, or pH which closely correlate to climatological or surface water conditions" (40 CFR 141.2).

The EPA revised the GWUDI source water classification to address contamination of groundwater sources by cryptosporidium (U.S. EPA, 2010). Giardia and cryptosporidium are protozoan parasites that are commonly found in human and animal waste (Hoogenboezem et al., 2001), and are a major cause of diarrhea worldwide, even in developed nations (Hancock et al., 1998; Striepen, 2013). Around 6% of the waterborne disease outbreaks in the USA from 1971 to 1996 were associated with giardia (U.S. EPA, 2000), and EPA rules for drinking water originally focused on removal of giardia. However, breakdowns in water treatment have been found to lead to very large outbreaks of cryptosporidiosis (MacKenzie et al., 1994; Widerstrom et al., 2014). The LT2 rule was designed to improve protection of drinking water supplies from cryptosporidium oocysts (U.S. EPA, 2010).

Microbial pathogens in surface waters, such as cryptosporidium, can be transported to hydraulically connected groundwater if natural filtration processes are inadequate (Hancock et al., 1998; U.S. EPA, 2010). Groundwater wells utilized as public water sources may potentially be misclassified as being groundwater rather than GWUDI, but the magnitude of the problem is unknown. The determination of GWUDI is based on the Microscopic Particulate Analysis (MPA) method which looks for evidence of surface biology (or surface physiochemical characteristics of water) in groundwater to assign an overall risk score for influence from surface water (U.S. EPA, 1992). The assumption is that the surface bioindicator groups are expected to occur in low numbers in groundwater that is not under direct influence from surface water. The determination is made more difficult because GWUDI characteristics may be seasonal or transient (U.S. EPA, 2010). The MPA method was designed to be a quick and inexpensive method to identify GWUDI, not to be an assay for cryptosporidium (U.S. EPA, 2010).

The detection and study of cryptosporidium oocysts has been hampered by the high costs and difficulty associated with producing and analyzing oocysts (Harter et al., 2000; Shin et al., 2001; Butkus et al., 2003; Ryan and Hijjawi, 2015). For many years, investigators have utilized microbial indicators for pathogens in drinking water (Payment and Locas, 2011 and references within), but the success of this approach has been variable at best. The EPA established *Escherichia coli* limits for water sources that if exceeded require sampling for cryptosporidium, but many studies have found no correlation between fecal indicators such as *E. coli* and cryptosporidium in water (Bonadonna et al., 2002; Harwood et al., 2005; Mons et al., 2009; Nieminski et al., 2010 and references within). Wilkes et al. (2009) found that the relationship between indicator bacteria, pathogens, and oocysts was positive, but overall weak, seasonally dependent, and site specific among water samples taken from river tributaries in Canada. Other studies have found that production of oocysts in animals is seasonal as well (Sturdee et al., 2003; Atwill et al., 2004; Dorner et al., 2004; Wilkes et al., 2009). Wu et al. (2011) examined relationship indicators and pathogens in the literature and found correlations in only 223 out of 540 cases. In some studies, non-enteric indicators have shown a greater correlation with pathogens then fecal indicators (Payment and Locus, 2011; Wu et al., 2011). The occurrence of fecal indicators in animals or the environment does not necessarily equate to an infection with cryptosporidium (Nieminski et al., 2010). Thus, pathogen monitoring of public water systems may not be as useful as originally thought and in the case of oocysts, relies on the incorrect assumption that oocysts are evenly distributed in samples (Allen et al., 2000). Cryptosporidium oocysts are only found in low concentrations in raw or treated waters when they are present (Cornwell et al., 2003; Guy et al., 2003; Horman et al., 2004; Briancesco and Bonadonna, 2005; Karanis et al., 2006; Brown and Cornwell, 2007) and for many public water systems, less than 0.001% of the water may actually be sampled for the presence of protozoans (Allen et al., 2000).

As such, the search for a cost effective and easily handled surrogate for cryptosporidium oocysts has been underway. Anaerobic (Schijven et al., 2003; Hinjen et al., 2007) and aerobic (Rice et al., 1994; Galofr et al., 2004; Mazoua and Chauveheid, 2005) bacterial spores, algae (Akiba et al., 2002; Hinjen et al., 2007), and microspheres (Dai and Hozalski, 2003; Emelko and Huck, 2004; Pang et al., 2012) have all been examined as possible surrogates for cryptosporidium oocysts. Aerobic bacterial spores are particularly good candidates because they are not pathogenic, can be produced and analyzed cheaply and easily in the laboratory, are persistent in the environment, and remain unchanged during transport, sampling, and laboratory analysis (U.S. EPA, 2010). The EPA suggests that aerobic bacterial spores be utilized as a surrogate for cryptosporidium (U.S. EPA, 2010).

Spore properties can vary tremendously between and within spore forming taxa (Kim et al., 2006; Driks et al., 2007). Consequently, not all aerobic bacterial spores are likely to be good candidates for use as a surrogate and some may only be suitable for specific purposes. The selection of an appropriate surrogate for cryptosporidium should involve a thorough examination of the biology and surface properties of both the pathogen and the proposed surrogate, as well as a consideration of the potential uses of

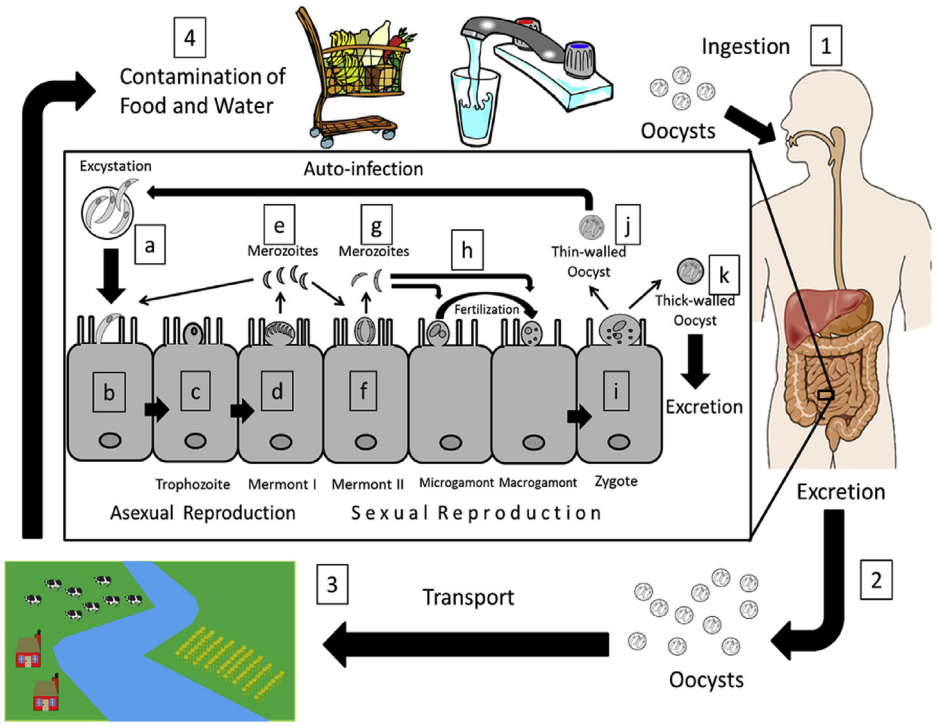

Fig. 1. Life cycle of cryptosporidium oocysts. **1**) Infection begins with the ingestion of oocysts. Once in the small intestine, **a**) the biochemical conditions induce the excystation of the Sporozoites within the oocysts. **b**) The Sporozoites enter epithelial cells and **c**) develop into Trophozoites. **d**) The Trophozoites develop into a type I Mermont and reproduce asexually to produce Merozoites. **e**) The Merozoites are released from the epithelial cells and either infect adjacent epithelial cells and reproduce asexually producing a trophozoite or **f**) the Merozoites infect epithelial cells and reproduced sexually and develop into a type II Mermont. **g**) The type II Mermont releases Merozoites from the epithelial cells which will infect epithelial cells and develop into either **h**) a Microgramont or a Macrogamont. The Microgamonts then fertilize the Macrogamonts to produce **i**) a Zygote. The Zygote then produces either **a** j) thin-walled oocysts which will remain in the host small intestine and lead to auto-infection or **k**) thick-walled oocysts which will be excreted. **2**). Mature oocysts are excreted from the host. **3**) The oocysts are transported in the environment eventually leading to **4**) contamination of food and water supplies which will be ingested by another host.

the surrogate (Sinclair et al., 2012). The use of aerobic spores as a surrogate for cryptosporidium to identify contaminated waters requires the consideration of environmental transport, survival, and prevalence properties. While, there have been numerous detailed studies of the transport behavior of oocysts (e.g. Kuznar and Elimelech, 2004, 2005, 2006; Tufenkji and Elimelech 2004a,b, 2005b, Kim et al., 2010b), there has been comparatively few studies examining the fate of aerobic bacterial spores (Pang et al., 1998; Chen et al., 2010).

The purpose of this paper is to provide a critical review of the literature with regard to the use of aerobic spores as a potential indicator of cryptosporidium in the determination of GWUDI and evaluation of bank filtration treatment. To this end, a comparison of the life cycles and anatomy of oocysts and spores will be presented. Next, we provide a description of the physical and surface properties, and interactions of this pathogen and surrogate. Literature information about the transport, survival, and prevalence of aerobic spores and oocysts in the environment is subsequently synthesized to assess whether aerobic spores are a suitable indicator of cryptosporidium in the environment, and to identify gaps in knowledge and future research directions.

2. Life cycle

In humans, Cryptosporidiosis is most commonly caused by the ingestion of oocysts produced by either *Cryptosporidium hominis*, which only infects humans, or *Cryptosporidium parvum* which is zoonotic (Robertson and Gjerde, 2007). Infective oocysts are shed into the environment through the feces of infected mammals (Harter et al., 2000; Petry, 2004) and theoretical calculations have estimated that a single cryptosporidium infection can produce as many as 2,000,000 oocysts a day (Upton, 2003; Robertson and Gjerde, 2007). The oocysts can survive in the environment for months, are resistant to conventional disinfection treatments, and highly infective, with as few as 10 oocysts capable of causing infection (Medema et al., 1998; Harris and Petry, 1999; Harter et al., 2000; Mazoua and Chauveheid, 2005).

Cryptosporidium infection (Fig. 1) begins with the ingestion of oocysts, which represent the dormant stage of cryptosporidium (Robertson and Gjerde, 2007). Each oocyst contains four banana shaped sporozoites and a residual body containing amylopectin which serves as an energy reserve for the sporozoites (Petry, 2004). The sporozoites only possess a single rhoptry and therefore are likely only capable of infecting a single epithelial cell (Petry, 2004). When exposed to bile salts, stomach acid, and amiable host temperatures, the oocysts cell wall breaks down and a suture in the wall opens that releases sporozoites that will infect intestinal epithelial cells of the host (Reduker et al., 1985; O' Donoghue, 1995; Harris and Petry, 1999). Once released from the oocyst, the sporozoite must infect a host cell quickly because it has no genes for the biosynthesis of amino acids, nucleotides, and sugars and therefore must rely on scavenging the host cell for nutrients (Abrahamsen et al., 2004; Petry, 2004). Inside the epithelial cells, the sporozoites develop into trophozoites that undergo asexual reproduction to produce type I meronts. The type I meronts produce merozoites that will either infect neighboring epithelial cells (to produce type I mermonts again) or develop into type II mermonts that will produce merozoites that undergo sexual reproduction to produce

microgamonts and macrogamonts (Petry, 2004). The microgamonts fertilize the macrogamonts to produce zygotes that will produce either thin-walled or thick walled oocysts (Hijjawi, 2010; Ryan and Hijjawi, 2015). Thin-walled oocysts will lead to autoinfection, while thick-walled oocysts are shed in the feces of the host (Current and Reese, 1986). Once shed in feces, the oocyst are infectious (Petry, 2004) and can be transported in the environment until they encounter their next host at which point the cycle will continue (Fig. 1).

While oocyst formation is part of the normal life cycle of cryptosporidium, bacterial sporulation is a last ditch effort by bacteria to survive in response to starvation (Stephens, 1998; Sonenshein, 2000; Driks, 2009; Leggett et al., 2012). The production of a spore is both time and resource consuming and bacteria that sporulate at the wrong time or when they are not fully capable are likely at a significant disadvantage (Parker et al., 1996). Before sporulation begins bacteria will attempt to activate flagella, produce antibiotics, secrete hydrolytic enzymes, and uptake exogenous DNA in an attempt to obtain nutrients (Stephens, 1998). If the chromosome is in sufficient condition and the bacterium possesses a functioning Krebs Cycle then the sporulation process can begin (Ireton et al., 1995; Grossman, 1995; Parker, 1996; Stephens, 1998). In general, lack of nutrients cause a drop in cellular Guanosine-5'-triphosphate (GTP) concentrations which leads to the expression of genes associated with sporulation (Sonenshein, 2000); however, the decision to sporulate is likely species specific and involves complex signal transductions that allow different taxa to exploit different environmental niches (Driks, 2009). Once a spore is produced, it can remain dormant for long periods of time (Kennedy et al., 1994 and references within) possibly millions of years (Cano and Borucki, 1995) until it encounters favorable environmental conditions (Leggett et al., 2012).

The sporulation process (Fig. 2) takes about 8 h (Driks, 1999, 2009) and is most efficient at high cell densities (Piggot, 1996). Sporulation begins when a normal vegetative cell undergoes asymmetric cell division to form a mother cell and a prespore. The mother cell then engulfs the prespore to produce a forespore. The point of no return is just before the prespore is engulfed by the mother cell. The forespore is bound by inner and outer membranes and peptidoglycan is produced between the inner and outer membranes to form what will become the spore cortex. The formation of the forespore is followed by the production of the spore coat and dipicolonic acid (DPA) which acts to chelate divalent ions in the core to reduce the water content within the spore. As the spore becomes more dense, the mother cell lyses and the mature spore is released into the environment (Parker et al., 1996; Driks, 1999; Paidhungat et al., 2000; Leggett et al., 2012).

Spores can be transported in the environment and will remain dormant until it encounters environmental cues to germinate. Germination is triggered by the presence of amino acids, sugars, nucleotides, calcium-DPA (Ca-DPA), and peptidoglycan fragments released from growing vegetative cells which bind to receptors in the inner membrane of the spore (Shah et al., 2008; Driks, 2009 and references within; Xiao et al., 2011) although, the exact signal to germinate is likely species and strain specific (Driks, 2009; Paredes-Sabja et al., 2011). The peptidoglycan produced in spores is different from that found in vegetative cells (Popham, 2002; Leggett et al., 2012). Spores will not germinate when they encounter peptidoglycan fragments from germinating spores, but they will sporulate when peptidoglycan from growing vegetative cells is released and binds to germinate receptors (Shah et al., 2008; Dworkin and Shah, 2010). Thus, spores use the presence of growing vegetative cells to sense the overall environmental conditions and determine when conditions are suitable to germinate. The implication is that spores will not germinate in any nutrient rich environment they encounter, but the environmental concentrations of germinants necessary to induce germination and the environments where germinates can persist long enough to stimulate germination are

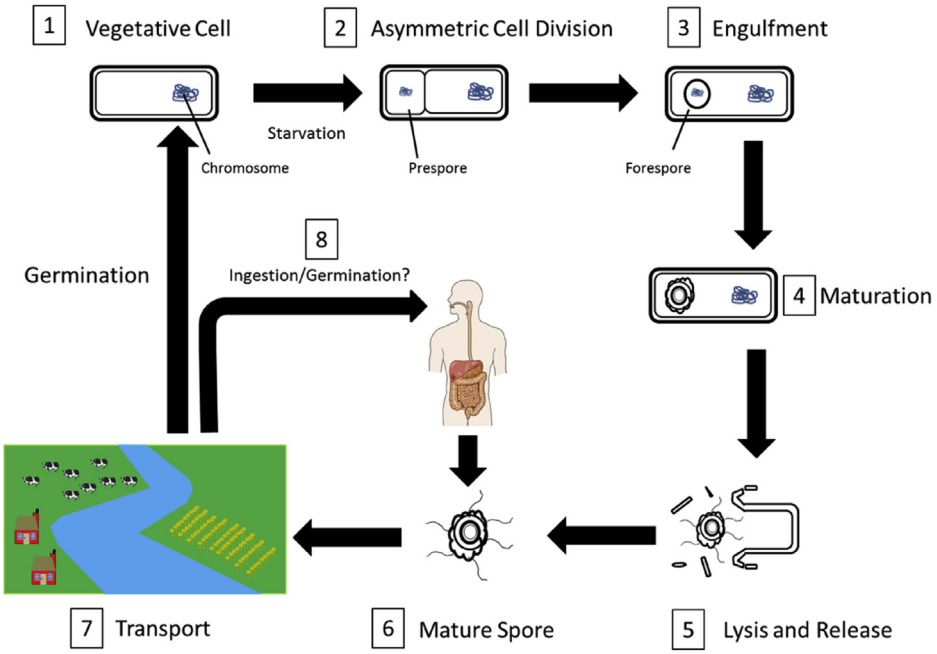

Fig. 2. The sporulation process. 1). A vegetative bacterial cell. 2) In response to starvation the cell undergoes asymmetric cell division to produce a prespore and a mother cell. 3) The prespore is engulfed by the mother cell and forms a forespore. 4) The spore matures and the various spore layers (cortex, coat, etc.) are produced and the core dehydrates. 5). The mother cell lyses and 6) the mature spore is released into the environment. 7) The spore is transported in the environment and will remain dormant until it encounters germinants in the environment, at which point the spore will germinate to produce a vegetative cell. 8) Some Bacillus spores may be ingested and germinate, proliferate, and re-sporulate in the small intestine before being excreted in feces back into the environment.

not clear (soils, stagnant waters, fast flowing rivers, etc.?). The number of germinant receptors in the inner membrane appears to vary by taxa. Spores of bacillus can have up to 8 different germinant receptors, while clostridium species appear to have less (Xiao et al., 2011). Low numbers of germinant receptors are associated with superdormant spores (Xiao et al., 2011; Chen et al., 2014).

While spore forming bacteria of the genus bacillus are often assumed to be soil bacteria, there is some evidence that bacillus spores might actually be gut commensals (Hong et al., 2009a, 2009b). Studies in mice have demonstrated that bacillus spores can germinate, proliferate, and re-sporulate anaerobically in the small intestine (Tam et al., 2006). While the concentration of recovered bacillus spores from feces samples is about 100 times less than that found in the soil (Hong et al., 2009b), the life cycles of bacillus spores and cryptosporidium oocysts may have much more in common than previously recognized. The revelation that bacillus spores pass through (and possibly are capable of residing in) the intestinal tract of humans and animals make bacillus spores excellent candidates for serving as a surrogate for cryptosporidium oocysts.

3. Anatomy

The inner portion of the oocyst contains the sporozoites, which infect intestinal epithelial cells, and the residual body which contains the energy reserves for the sporozoites while they remain in the oocyst (Fig. 3). The sporozoites are surrounded by a tri-layered oocyst wall approximately 40–55 nm thick (Harris and Petry, 1999; Jenkins et al., 2010). The inner portion of the oocyst wall is composed of filamentous glycoprotein rich in cysteine and disulfide bonds that impart structural stability to the wall (Ranucci et al., 1993; Spano et al., 1997; Harris and Petry, 1999; Bushkin et al., 2013). The central portion of the wall is composed of complex lipids, mostly triglycerides with long fatty acid acyl chains, that also impart some rigidity to the wall (Harris and Petry, 1999; Bushkin et al., 2013). The outer wall is composed of acidic glycoprotein and is surrounded by a large glycocalyx composed of glucose, galactose, mannose, xylose, and ribose (Reduker et al., 1985; Harris and Petry, 1999; Nanduri et al., 1999; Jenkins et al., 2010) and anchored glycoproteins and acidic macromolecules (Harris and Petry, 1999; Considine et al., 2002; Kuznar and Elimelech, 2005, 2006; Jenkins et al., 2010). A linear suture spans from one-third to one-half the surface of the oocyst wall (Reduker et al., 1985) and splits to allow the sporozoites to exit when exposed to appropriate environmental cues (Petry, 2004).

The interior anatomy of aerobic bacterial spores (Fig. 4) is generally similar across taxa, but the dimensions and properties of spores can vary considerably among taxa depending on growth and environmental conditions (Plomp et al., 2005; Driks, 2009). The innermost portion of the spore is the core which contains the crucial biochemical components of the cell (DNA, ribonucleic acid, etc.). The core is also heavily mineralized and contains large concentrations of divalent ions, calcium-DPA (Ca-DPA), and small acid-soluble spore proteins that bind directly to the DNA to prevent damage from chemicals and radiation (Marquis and Bender, 1985; Beaman and Gerhardt, 1986; Setlow, 2006; Sunde et al., 2009; Leggett et al., 2012). The core contains very little water (Beaman et al., 1982) and the water permeability in the core is two orders of magnitude lower than typical cellular membranes (Sunde et al., 2009). The core is surrounded by a low permeability inner membrane that contains the receptors for germinants (Setlow, 2006; Leggett et al., 2012). The low permeability appears to be due to compressed, immobile lipids and the low water content of the spore (Cowan et al., 2004). Surrounding the inner membrane is a germ cell wall that will become the bacterial cell wall when the spore germinates (Setlow, 2006; Henriques and Moran, 2007).

Exterior to the germ cell wall is the cortex, which is composed of peptidoglycan that is different from that found in vegetative cell walls (Popham, 2002; Leggett et al., 2012). The peptidoglycan of *Bacillus subtilis* spores is unique in that the N-acetylmuramic acid residues do not contain any teichoic acids and there is a reduction in cross-linking (Popham, 2002). In addition, most spores contain a meso-diaminopimelic acid residue instead of an L-Lysine residue in the stem peptide of peptidoglycan (Shah et al., 2008). Surrounding the cortex is the outer membrane, which does not appear to be a significant permeability barrier or act as a functional membrane (Setlow, 2006; Leggett et al., 2012). Surrounding the outer membrane is the coat, which provides the spore with protection from chemicals and enzymes (Driks, 1999, 2002; Henriques and Moran, 2007; Leggett et al., 2012) and acts as a barrier to large molecules, while allowing the smaller germinant molecules to pass (Driks, 1999, 2002; Leggett et al., 2012). The coat is comprised primarily of proteins with minor amounts of carbohydrates (Driks, 1999; Leggett et al., 2012). The composition, architecture, and thickness of the spore coat varies within and between species and there is a high degree of disorder in the coat (Driks, 1999; Kim et al., 2006; Henriques and Moran, 2007; Leggett et al., 2012). The variation in spore coats among different species is due to species specific crystallization mechanisms that regulate spore coat assembly (Plomp et al., 2005). The most prominent structure on the spore coat are ridges that fold and unfold to allow the spore to accommodate changes in volume in response to changing environmental conditions such as humidity (Chada et al., 2003; Westphal et al., 2003; Plomp et al., 2005; Kim et al., 2006). The ridges disappear as the spores swell in response to diffusion of water into the spore coat (Westphal et al., 2003; Chada et al., 2003). The ridges of *B. subtilis* spores are approximately 12 nm in height and 85 nm thick along the long axis of the spore (Chada et al., 2003).

The exosporium is an optional feature for some bacterial spores and is the outermost layer for bacterial spores that possess an exosporium (Setlow, 2006; Giorno et al., 2007; Driks, 2009; Leggett et al., 2012). The structure and composition of the exosporium is species specific (Henriques and Moran, 2007). *B. subtilis* spores either do not have an exosporium or it is tightly attached (Waller et al., 2004; Setlow, 2006). The exosporium is composed mainly of glycoprotein with low levels of amino acids containing sulfur, some lipids, carbohydrates, and ash (Matz et al., 1970; Sylvestre et al., 2002; Leggett et al., 2012). The exosporium is separated from the coat by an uncharacterized region referred to as the interspace (Giorno et al., 2007; Driks, 2009). Many proteins on the exosporium surface have been found not to be components of the exosporium and may be contaminant proteins captured by the exosporium (Tauveron et al., 2006).

Appendages are not present on all spores and the structure (pilus-like, ribbon-like, etc.), number and length of appendages varies among species and strain (Tauveron et al., 2006; Walker et al., 2007). Appendages are mostly made of proteins and they can be up to micrometers in length (Stalheim and Granum, 2001; Tauveron et al., 2006). The function of the appendages appears to be to assist in adhesion in specialized or high flow environments by overcoming initial barriers to attachment where it is difficult for spores to adhere (Husmark and Ronner, 1992; Stalheim and Granum, 2001; Driks, 2007), however, once attached to the surface, the appendages appear to serve no further purpose (Klavenes et al., 2002).

4. Preparation of oocysts and aerobic spores in the laboratory

Oocysts propagation in the laboratory environment is difficult, typically requiring special facilities and trained personnel to safely

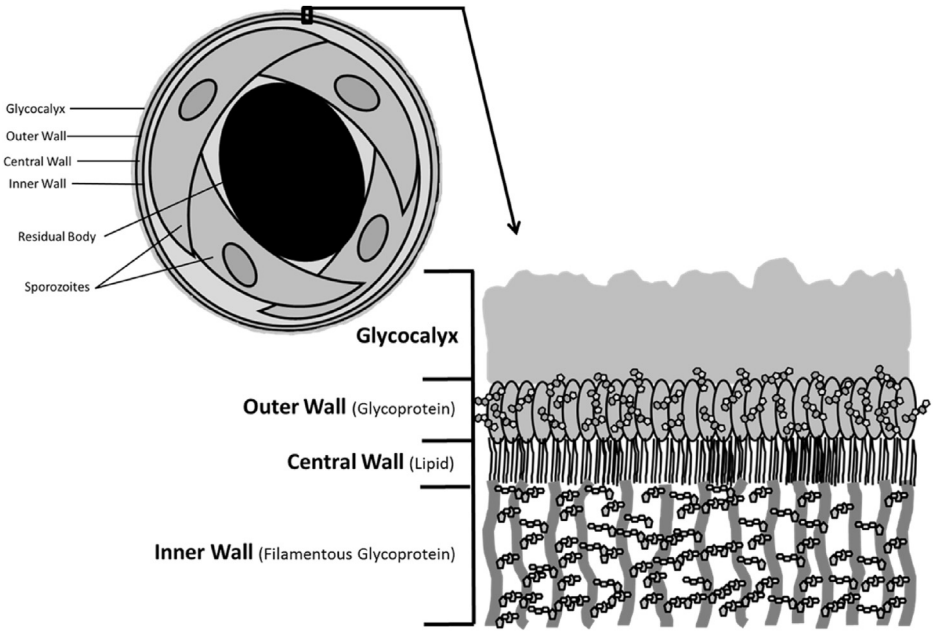

Fig. 3. The anatomy of a *Cryptosporidium* oocyst. Four banana shaped sporozoites surround a residual body containing nutrients for the sporozoites survival. The oocyst wall consist of an inner wall composed of filamentous glycoprotein, a central wall composed of lipids, an outer wall composed of glycoproteins, and a glycocalyx surrounding the outer wall.

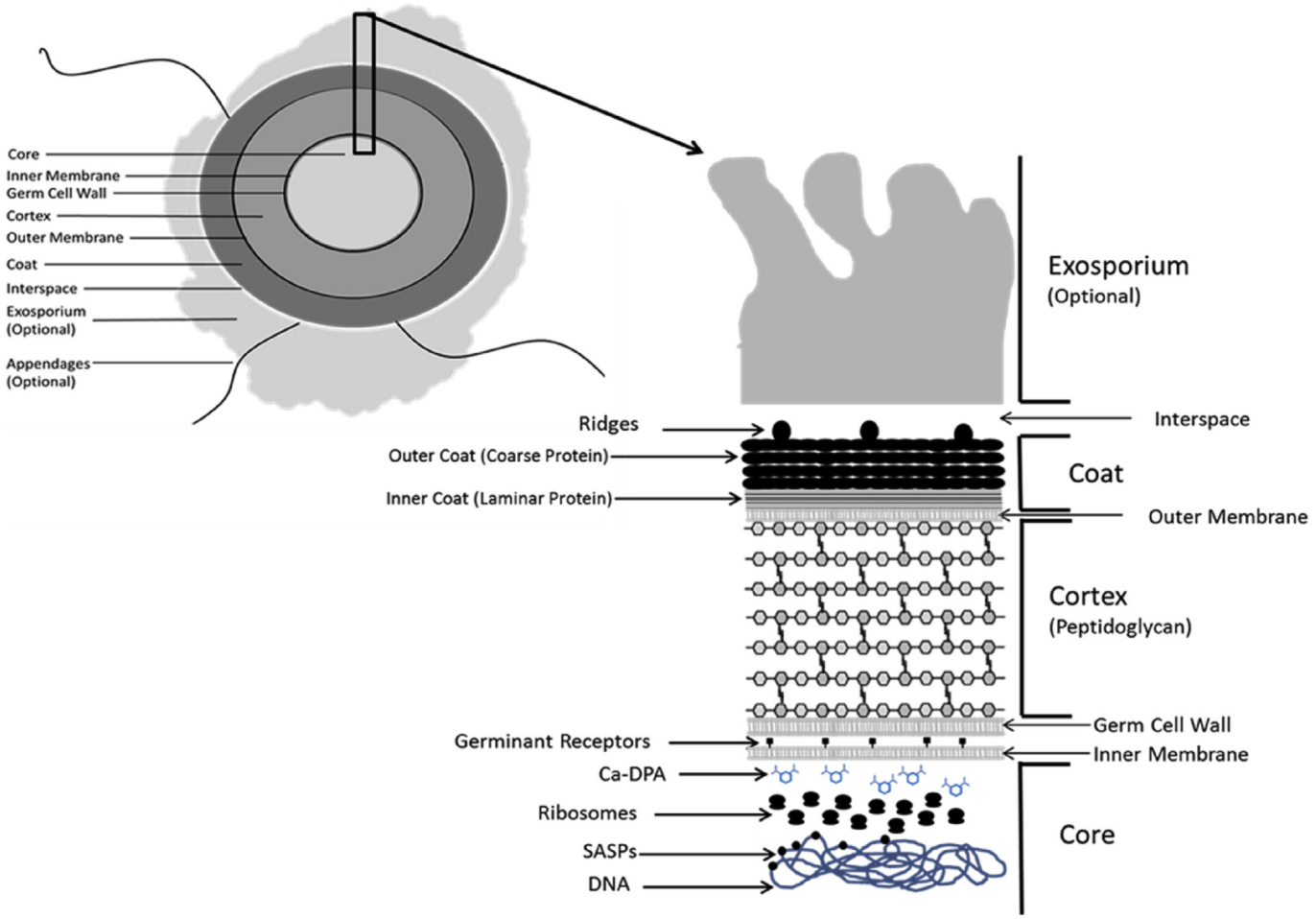

Fig. 4. The anatomy of a bacterial spore. The innermost portion consists of a dehydrated core which contains all of the necessary cellular biochemical components (DNA, RNA, enzymes, etc.) Surrounding the core is an inner membrane and a germ cell wall. The germ cells wall will develop into the cell wall when the spore germinates. Surrounding the germ cell wall is the cortex, an outer membrane and a coat. Some bacteria spores possess an exosporium, separated from the coat by an interspace region, and/or appendages.

produce large numbers of oocysts (Petry et al., 1995). Oocysts can be propagated by passage through pathogen-free animals models such as calves, lambs, pigs, and rodents and then recovered in the feces, although more recently various cell cultures and cell-free culture media have become available (Petry et al., 1995; Suresh and Rehg, 1996; Arrowood, 2002 and references within; Enemark et al., 2003; Hijjawi et al., 2004; Ware and Villegas, 2010). The number of oocysts generated varies depending on the animal model (Arrowood, 2002) and the reproducibility of oocysts in cell culture and cell free culture media has been an issue (Hijjawi, 2010). Once the oocysts are produced they must be isolated and purified, typically by sucrose, cesium chloride, or Percoll gradient separation, ion exchange column chromatography, passage through glass beads, filtration, and/or dialysis (Suresh and Rehg, 1996; Arrowood, 2002 and references within). For those researchers without the facilities to produce oocysts in house, oocysts can also be purchased from various commercial vendors and university labs.

By contrast, aerobic spore propagation can cheaply and easily be performed in most laboratories. The most commonly used method for spore production is nutrient exhaustion, but there are a variety of other methods (and variations in the nutrient exhaustion method) that have been developed to suit the different research goals of investigators (Nicholson and Setlow, 1990). Nutrient exhaustion simply requires the spore forming bacterial strain to be grown in an appropriate media for sufficient time to allow for the nutrients to be exhausted and starvation and subsequent spore production to ensue. Spore production can be monitored via microscopy of the culture and high spore yields can typically be produced in a matter of days depending on the growth characteristics of the strain. The spores are typically harvested by several rounds of centrifugation and resuspension in cold water (or other desired solution) to separate the spores from the growth media (Nicholson and Setlow, 2000).

One issue that has been largely ignored by many researchers is the purity of spore preparations being used in spore studies. Every spore generated came from a cell that has lysed and released its contents into the growth medium. Cellular debris adheres to spore surfaces (Goss et al., 2015). Despite the existence of an array of different methods to purify spores (Sacks and Alderton, 1961; Jenkinson et al., 1981; Nicholson and Setlow, 1990; Weincek et al., 1990; Doyle et al., 1984; Brahmbhatt et al., 2007; Harold et al., 2011), many studies fail to report if or how spores were actually purified (Tavares et al., 2013). Most studies appear to regard purification as simply the removal of vegetative cells and make no active attempt to remove cellular debris (Zhao et al., 2008). The most common purification method appears to be the repeated washing of spore preparations in cold deionized water followed by centrifugation (e.g. Koshikawa et al., 1989; Wiencek et al., 1990; Ahimou et al., 2001; Chen et al., 2010; Faille et al., 2010), but this will not completely separate attached debris from the spores (Goss et al., 2015). Equally suspect is microscopic determination of purity because the majority of the cellular debris generated during sporulation is too small to be viewed in a microscope (i.e. proteins, nucleic acids, etc.). Any cellular debris attached to spore surfaces would alter spore properties (surface chemical and physical properties, buoyant density, etc.) and would certainly impact zeta potential and hydrophobicity measurements, as well as, subsequent modeling. Purification treatments with heat or lysozyme are known to alter spore surface properties and affect surface hydrophobicity (Doyle et al., 1984; Craven and Blankenship, 1987; Wiencek et al., 1990; Zhao et al., 2008). To our knowledge, the issue of spore purity has only been examined recently in a handful of studies (Dragon and Rennie, 2001; Zhao et al., 2008; Harrold et al., 2011; Tavares et al., 2013; Goss et al., 2015) but, these studies make a strong case for researchers to evaluate their purification procedures. In particular, many commonly used purification procedures appear to be strain specific and small adjustments to these protocols can yield different (better) results.

5. Physical characteristics

One of the most prominent differences between oocysts and aerobic spores is their respective size and densities (Table 1). The *C. parvum* oocyst is spherical to ellipsoidal in shape and varies in size from approximately 3.5 to 6.0 μm in diameter (Reducker et al., 1985; Medema et al., 1998; Robertson and Gjerde, 2007). The density of oocysts has been reported to be 1.045 g/cm^3 (Medema et al., 1998); however, in aquatic environments the density can vary due to depletion of the internal resources associated with aging of the sporozoites and environmental stressors (Medema et al., 1998). Aerobic bacterial spores tend to range from about 1.0 to 2.0 μm in length and about 0.8 μm in width depending on species and strain (Mamane Gravetz and Linden, 2005, Plomp et al., 2005; Blanpain-Avet et al., 2011; Fricker et al., 2011). The density of *B. subtilis* spores varies between 1.290 g/cm^3 for light spores to 1.335 g/cm^3 for heavy spores (Dean and Douthit, 1974). Light spores arise earlier during sporulation and have less calcium, potassium, manganese, and DPA in their cores and more water and sodium in their cores than heavy spores (Dean and Douthit, 1974). Light spores may represent immature spores (Dean and Douthit, 1974). The density of other Bacillus species varies by about 0.2 g/cm^3 relative to one another (Beaman et al., 1982).

6. Surface properties

6.1. Charge

A wide range of oocysts zeta potentials have been reported in a variety of solutions (e.g. Hsu and Huang, 2002; Butkus et al., 2003; Kuznar and Elimelech, 2006). Oocysts surface charges are generally neutral to slightly negative above the isoelectric point (~pH 2.5) and remain negative to pH 12 (Drozd and Schwartzbrod, 1996; Karaman et al., 1999; Harter et al., 2000; Butkus et al., 2003; Tufenkji et al., 2006). The zeta potential of oocysts become less negative with increasing ionic strength and decreasing pH due to acidic functional groups and/or phosphates groups on the surface (Karaman et al., 1999; Hsu and Huang, 2002; Kuznar and Elimelech, 2005, 2006; Tufenkji and Elimelech, 2005a; Tufenkji et al., 2006; Kim et al., 2010b) and compression of the double layer (Butkus et al., 2003). However, binding of multivalent cations to functional groups on the oocyst surface can lead to charge neutralization and/or reversal, and a reduction in zeta potentials (Kuznar and Elimelech, 2004; Tufenkji et al., 2006). Furthermore, the surface charge of *C. parvum* oocysts may be altered by washing with deionized water or low ionic solvents that can remove the glycocalyx (Butkus et al., 2003; Jenkins et al., 2010).

Similar to oocysts, the isoelectric point of many aerobic spores falls below pH 3 (White et al., 2012) and the zeta potential of spores become less negative with increasing ionic strength and decreasing pH (Kuznar and Elimelech, 2004; Seale et al., 2008). Spores with similar structures (exosporium, appendages, etc.) tend to have similar zeta potentials (White et al., 2012), but large variations in zeta potentials and electrophoretic mobilities exists among aerobic bacterial spore measurements (e.g., Ahimou et al., 2001; White et al., 2012). In general, aerobic spores have a more negative surface potential than oocysts, but this to can vary depending on spore strain and solution chemistry (e.g., Ahimou et al., 2001; Faille et al., 2010). To date, there have been very few direct comparisons between oocyst and aerobic spore surface charge. Furthermore, differences in zeta potentials of cryptosporidium oocysts and aerobic

Table 1
A comparison of oocysts and aerobic spores properties.

	Oocysts	Aerobic spores
Size[a]	3.0–6.0 μm	1.0–2.0 μm
Density[b]	1.045 g/cm^3	1.290–1.355 g/cm^3
Chemical Susceptibility[c]	Ammonia, Chlorine dioxide, Ethylene oxide, Hydrogen peroxide, Methyl bromide, Ozone	Calcium hypochlorite, Chlorine dioxide, Ethylene oxide, Free chlorine, Formaldehyde, Glutaldehyde, Hydrogen peroxide, Iodine compounds, Methyl bromide, Nitrous acid, Ozone, Beta-Propiolactone, Peracetic acid, Propylene oxide, Sodium hydroxide, Sodium hypochlorite
UV resistance[d]	Sensitive at 10–30 J/m^2	LD90 = 113.7 J/m^2 in air-dried monolayers to 156 J/m^2 in aqueous suspension
Cold resistance[e]	−22 °C	−78 °C
Heat resistance[f]	Up to 55 °C	>100 °C
Desiccation[g]	Lethal	Resistant
Dormancy[h]	Months	Years
Environmental prevalence[i]	Low concentrations in waters, high concentrations near animal operations, seasonal	Ubiquitous, can exhibit seasonality near animal operations
Hydrophobicity[j]	Generally hydrophilic	Hydrophilic to hydrophobic
Isoelectric Pont[k]	~pH 2.5	<pH 3
Zeta potential[l]	Generally less negative than aerobic spores, but can vary considerably. More negative with increase in pH and decrease in ionic strength	Generally more negative than oocysts, but can vary considerably. More negative with increase in pH and decrease in ionic strength

[a] Reducker et al., 1985; Medema et al., 1998; Robertson and Gjerde, 2007; Mamane Gravetz and Linden, 2005, Plomp et al., 2005, Blanpain-Avet et al., 2011, Fricker et al., 2011.
[b] Medema et al., 1998; Dean and Douthit, 1974.
[c] Fayer et al., 1996; Peeters et al., 1989; Korich et al., 1990; Weir et al., 2002; Russell, 1990; Whitney et al., 2003 and references within; Setlow et al., 2006.
[d] Shin et al., 2001; Xue and Nicholson, 1996; Nicholson et al., 2000.
[e] Robertson et al., 1992; Fayer et al., 1998; Nicholson et al., 2000.
[f] Fujino et al., 2002; Nicholson et al., 2000.
[g] Robertson et al., 1992; Nicholson et al., 2000.
[h] Robertson et al., 1992; Harris and Petry, 1999; Kennedy et al., 1994 and references within; Cano and Borucki, 1995.
[i] Kauri, 1982; Phillips and Griffiths, 1986; Slaghuis et al., 1997; Nicholson et al., 2000; Cornwell et al., 2003; Brown and Cornwell, 2007; Sturdee et al., 2003; Dorner et al., 2004; Wilkes et al., 2009; Buehner et al., 2014.
[j] Drozd and Schwartzbrod, 1996; Kuznar and Elimelech, 2005, 2006; Doyle et al., 1984, Koshikawa et al., 1989; Ahimou et al., 2001; Faille et al., 2010.
[k] Drozd and Schwartzbrod, 1996; Karaman et al., 1999; White et al., 2012.
[l] Drozd and Schwartzbrod, 1996; Karaman et al., 1999; Hsu and Huang, 2002; Lytle et al., 2002; Kuznar and Elimelech, 2004; Tufenkji et al., 2006; Ahimou et al., 2001; Lytle et al., 2002, White et al., 2012.

spores can also be difficult to interpret because of the wide variety of solution chemistries used in measurements. The best solution is likely a direct comparison of measured zeta potentials for oocysts to potential aerobic spore surrogates to ensure that similar solution chemistries are utilized in measurements. Tables S1 and S2 present a summary of some published zeta potentials for oocysts and aerobic spores, respectively.

6.2. Hydrophobicity

Oocysts are generally hydrophilic (Kuznar and Elimelech, 2005, 2006) (Table 1), but the hydrophobicity of oocysts changes with the age of the oocyst and after they are excreted into the environment due to physical and chemical changes in the oocyst wall (Brush et al., 1998). Hydrophobicities increase towards extremes in pH, but are minimal at pH 7 (Drozd and Schwartzbrod, 1996; Hsu and Huang, 2002). Solution conductivity can cause changes in surface hydrophobicity as well (Drozd and Schwartzbrod, 1996).

The hydrophobicity of aerobic spores is greater than vegetative cells (Andersson and Ronner, 1998; Ronner et al., 1990; Wiencek et al., 1990; Ahimou et al., 2001) due to the abundance of proteins in spore coats and the exosporium compared to the abundance of peptidoglycan found in many spore forming bacteria cell walls (Matz et al., 1970; Takumi et al., 1979; Doyle et al., 1984; Wiencek et al., 1990). Large variations in hydrophobicities have been measured among taxa due to variations in the composition of the outer surfaces (coat, exosporium, appendages) (Doyle et al., 1984; Koshikawa et al., 1989; Ronner et al., 1990; Andersson and Ronner, 1998; Ahimou et al., 2001; Tauveron et al., 2006), the conformation of which can change in response to pH, heat, protein denaturants, and enzymes (Doyle et al., 1984; Wiencek et al., 1990; Seale et al., 2008). Spores with an exosporium are generally more hydrophobic than those without an exosporium (Koshikawa et al., 1989). Sporulation medium and temperature has been shown to generally not affect Bacillus spore hydrophobicity (Wiencek et al., 1990). In general, aerobic spores tend to be more hydrophobic than oocysts (Table 1), but some spore strains (for example *B. subtilis* B213; ATCC 15476, and ATCC 6051) have been reported to be hydrophilic (Ahimou et al., 2001; Koshikawa et al., 1989) and there are almost certainly many others. Tables S3 and S4 present a summary of some published hydrophobicity measurements for oocysts and aerobic spores, respectively.

Some hydrophobicity assays measure the ability of microbes to bind to a hydrophobic surface (e.g., microbial adhesion to hydrocarbon, MATH; and hydrophobic interaction chromatography, HIC), while others measure the overall surface hydrophobicity (contact angle measurements) (Van der Mai et al., 1987). These hydrophobicity assays are extremely susceptible to methodology, not just biochemical differences among microbes, because they measure different properties (Mozes and Rouxhet, 1987; Van der Mei et al., 1987). For example, variations in vortex duration and intensity, the hydrocarbon used, ratio of hydrocarbon to aqueous solution, presence of hydrocarbon droplets, purity of hydrocarbon solutions, temperature, and the chemical makeup of the aqueous solution can have a significant impact on the results of the widely used MATH assay (Dillon et al., 1986; van der Mei et al., 1987; Koshikawa et al.,

1989; Rosenberg, 1991, 2006; Hori et al., 2008; Saini, 2010; Zoueki et al., 2010). It is therefore difficult to quantitatively compare the results from different studies, even when the same organism is examined. Thus, a simple literature review of measured spore hydrophobicities is unlikely to be an effective strategy to identify a suitable surrogate for oocysts. However, there exists good agreement among the various hydrophobicity methods in identifying general trends in hydrophobicity and in making qualitative comparisons between spores of different species and strains (Mozes and Rouxhet, 1987; Wiencek et al., 1990). It has been recommended that a combination of assays be used to obtain hydrophobicity information (Dillon et al., 1986; Mozes and Rouxhet, 1987; Van der Mai et al., 1987), but a more useful approach might be to make direct comparisons between hydrophobic measurements of oocysts and potential surrogates.

7. Surface interactions

Oocysts and spores experience attractive or repulsive interaction energies as they approach an interface. Theory developed by Derjaguin-Landau-Verwey-Overbeek (DLVO) is commonly employed to determine the interaction energy that arises from the superposition of electrostatic and van der Waals interactions (Derjaguin and Landau, 1941; Verwey and Overbeek, 1948). Various approximations of the Poisson–Boltzmann equation are used to account for the electrostatic interactions between charged bodies in ionic solutions as a result of the overlap of their diffuse double layers (Elimelech et al., 1995). These expressions employ information about the double layer thickness (solution ionic strength) and surface charge (zeta potentials). The electrostatic interaction is repulsive when both surfaces are negatively charged, and it is attractive when the surfaces are of opposite charge. Attractive van der Waals interactions occur between charged surfaces due to the presence of intermolecular forces that occur as a result of polarization of molecules into dipoles (Israelachvili, 1992). Other non-DLVO interactions may occur due to Born repulsion, hydrophobic forces, and steric interactions (Israelachvili, 1992; Elimelech et al., 1995). Born repulsion is a strong short ranged force that occurs due to the size of atoms and their electron shells. Hydrophobic forces are dependent on interfacial Lewis acid-base and van der Waals interactions (van Oss et al., 1986; van Oss et al., 1987; van Oss, 1994; van Oss, 2003). Steric interactions result from the sorption of chains and/or chain elements onto surfaces (Rijnaarts et al., 1999). The magnitude of steric forces depends on the inter-chain strand changes in osmotic pressure, chain length, charge, and elasticity (Bowen and Williams, 1995; Ohshima, 1995).

Hydrophobic interactions are expected to play an important role between low energy (hydrophobic) surfaces such as between hydrophobic spores and the air–water interface, other spores, and some types of organic matter. The hydrophobic properties of many aerobic (and anaerobic) spores have been recognized to be important factors behind spore adhesion to surfaces (Husmark and Ronner, 1990, 1992, 1993; Faille et al., 2010). Greater hydrophobicity generally corresponds to a greater ability of spores to attach to surfaces (Andersson and Ronner, 1998; Ronner et al., 1990). However, most studies have examined spore hydrophobicity in the context of removing spores from surfaces in industrial environments (e.g. Parkar et al., 2001; Faille et al., 2002; Klavenes et al., 2002; Tauveron et al., 2006; Faille et al., 2010; Harimawan et al., 2013), not subsurface migration. The hydrophobicity of the exosporium and its proteins has been reported to allow spores to adhere to surfaces (Koshikawa et al., 1989; Tauveron et al., 2006; Brahmbhatt et al., 2007; Henriques and Moran, 2007; Driks, 2009). All spores of *Bacillus cereus* possess a hydrophobic exosporium and they have been shown to strongly attach to stainless steel, polymers, and glass (Husmark and Ronner, 1992).

Steric interactions are expected to be important in the presence of significant amounts of microbial surface macromolecules and structures. The glycocalyx surrounding the oocyst forms a brush like surface that has been reported to cause steric repulsion with solid surfaces (Considine et al., 2002; Kuznar and Elimelech, 2005, 2006; Jenkins et al., 2010). Kuznar and Elimelech (2006) removed the surface glycoproteins from oocysts and found that the zeta potential become more negative and the oocysts hydrophobicity decreased slightly while attachment efficiency to quartz increased. The authors attributed this behavior to reduced steric repulsion on the oocyst surface caused by the removal of charged functional groups (Kuznar and Elimelech, 2006). In unmodified oocysts, the steric repulsion causes reduced attachment at higher ionic strengths (Considine et al., 2000; Kuznar and Elimelech, 2004, 2005, 2006) even when DLVO theory predicts no energy barrier (Kuznar and Elimelech, 2004, 2005; Kim et al., 2010b). Other studies have now emphasized the importance of steric repulsion in the attachment of bacteria and oocysts (Kim et al., 2009, 2010b; Liu et al., 2009). The possible influence of steric repulsion on aerobic spore interactions has not yet been studied.

Natural surfaces always exhibit some degree of nanoscale roughness (Suresh and Walz, 1996). Nanoscale roughness can have a pronounced influence on interaction energy profiles due to the different functional dependence of electrostatic and van der Waals interactions with separation distance (Hoek et al., 2003; Huang et al., 2010; Bendersky and Davis, 2011). Nanoscale roughness tends to produce a finite depth of the primary minimum, a reduction or elimination of the local energy barrier, and a diminished depth of the secondary minimum (Shen et al., 2012; Bradford and Torkzaban, 2013). However, the extent of the modification to the interaction energy profile depends on the local roughness height and density, with more primary minimum interactions occurring at lower roughness densities (Torkzaban and Bradford, 2016). Similarly, the morphological properties on spore surfaces (such as appendages) have been reported to assist in adhesion by overcoming initial barriers to attachment (Husmark and Ronner, 1990, 1992; Stalheim and Granum, 2001; Faille et al., 2002; Klavenes et al., 2002; Driks, 2007; Seale et al., 2008). The presence of spore appendages has been positively correlated to spore attachment to stainless steel (Klavenes et al., 2002; Tauveron et al., 2006). The role of nanoscale roughness on oocyst interactions has not been explicitly studied, but it is likely related to the reported effects of steric repulsion discussed above.

Nanoscale chemical heterogeneity may occur on the surfaces of microbes and soils as a result of protonation and deprotonation of surface hydroxyl groups, and adsorption of multivalent ions and organics (Vaidyanathan and Tien, 1991; Tufenkji and Elimelech, 2005a). Heterogeneity will also undoubtedly be introduced on the surfaces of spores and oocysts as a result of: incomplete removal of organics during purification; variations in the amount and conformation of surface macromolecules and/or appendages; differences in the surface hydrophobicity and calcium content; and population heterogeneity arising from species, age, and genetic diversity (Bolster et al., 2009; 2010). Nanoscale chemical heterogeneity can locally reduce or eliminate the energy barrier to attachment (Duffadar and Davis, 2008; Bendersky and Davis, 2011). The influence of chemical heterogeneity on interaction energy profiles has been reported to be dependent on the heterogeneity size, amount, and zeta potential, the microbe size, and the solution ionic strength (Bradford and Torkzaban, 2012, 2013). More primary minimum interactions occur for smaller microbe size, higher ionic strengths, and for heterogeneities that are larger in size and/or more frequent, and with greater positive charge (Bradford and Torkzaban, 2012, 2013).

8. Subsurface migration

Oocysts are typically found in low concentrations in natural aqueous environments (Cornwell et al., 2003; Guy et al., 2003; Horman et al., 2004; Briancesco and Bonadonna, 2005; Karanis et al., 2006; Brown and Cornwell, 2007). Consequently, most transport studies with oocysts have been conducted at the laboratory scale (e.g., Tufenkji et al., 2006). In contrast, there have been numerous studies examining spore removal in various water treatment processes (Barbeau et al., 1999; Facile et al., 2000; Cornwell et al., 2003; Galofr et al., 2004; Weiss et al., 2005; Brown and Cornwell, 2007), but detailed transport studies that have been performed for oocysts are largely lacking for aerobic bacterial spores. Available literature on the transport, retention, and release of oocysts and spores in the environment will be briefly highlighted below. Note that mathematical models to describe microbial transport and survival in the subsurface environment will not be discussed in detail, as this was the topic of another recent review (Bradford et al., 2014).

8.1. Transport

Oocysts and spores are transported in aqueous subsurface environments as a result of advection, diffusion, and mechanical dispersion. These microbes can be transported freely in water (Dai and Boll, 2003) or attached to other particles (Medema et al., 1998). Size and/or anion exclusion of oocysts and spores from smaller portions of the pore space produces a velocity enhancement relative to a conservative solute tracer (Mawdsley et al., 1996; Pang et al., 1998; Sinton et al., 2000; Harter et al., 2000; Tufenkji et al., 2004; Hijnen et al., 2005). Greater amounts of size exclusion and velocity enhancement are expected for larger microbes (Ginn, 2002; Bradford et al., 2003; Scheibe and Wood, 2003). Preferential flow in macropores has also been demonstrated to rapidly transport oocysts through soils and minimize retention (Darnault et al., 2003, 2004). In stagnant water, microbes can settle and accumulate in sediment. The sedimentation rate depends on the microbe size, density, and association with particles (Medema et al., 1998; Hijnen et al., 2005). Spores that aggregate will have greater sedimentation rates (Husmark and Ronner, 1990). The sedimentation velocity for oocysts has been reported to increase by a factor of 50 when associated with inorganic particles (Searcy et al., 2005).

8.2. Retention

Higher clay, metal oxide, and organic content in soils lead to more attachment of oocysts onto soil particles (Mawdsley et al., 1996; Hijnen et al., 2005; Abudalo et al., 2005; Mohanram et al., 2010; Balthazard-Accou et al., 2014). Furthermore, greater oocysts attachment occurs at lower velocities (Harter et al., 2000; Kim et al., 2010b) and higher ionic strengths (Tufenkji et al., 2004; Tufenkji and Elimelech, 2005a; Kim et al., 2010b). In monovalent salt solutions, increased attachment of oocysts to surfaces at higher ionic strength is attributed to compression of the double layer (Kuznar and Elimelech, 2004; Tufenkji et al., 2004; Tufenkji and Elimelech, 2005b). In divalent solutions, the binding of divalent ions to surface functional groups can neutralize surface charges and induce conformation changes in surface proteins that can alter oocyst attachment (Kuznar and Elimelech, 2004; Janjaroen et al., 2010). Changes in the properties of the oocyst wall as they age have been shown to affect adhesion and transport properties of oocysts in natural environments (Robertson et al., 1992; Brush et al., 1998). Oocyst removal by slow sand filtration has been reported to decrease at lower temperatures (Fogel et al., 1993).

Straining also plays an important role in oocysts retention during infiltration and recharge, riverbank filtration, and slow sand filtration (Tufenkji et al., 2004; Hijnen et al., 2005; Bradford and Bettahar, 2005; Kim et al., 2010b). Oocysts tend to be retained by the finer fractions of sediments (Bradford and Bettahar, 2005; Harter et al., 2000; Hijnen et al., 2005; Balthazard-Accou et al., 2014) and in low velocity regions (Kim et al., 2010b). The transport and retention of oocysts is very sensitive to the grain size (Kim et al., 2010b), but grain size alone is not always a good predictor of microbial transport in natural soils (Hinjen et al., 2005). Grain angularity and roughness also have been reported to influence oocyst straining (Tufenkji et al., 2004). Bradford and Torkzaban (2015) found that surface straining at microscopic roughness locations increased with water velocity, colloid size, and decreasing solution ionic strength.

In comparison to oocysts, relatively few systematic studies have been conducted to investigate spore retention. Chen et al. (2010) studied the transport of various species of *B. subtilis* and *Bacillus anthracis* spores in unsaturated column experiments. These authors related differences in the transport behavior to variations in the spore anatomy, surface charge, and hydrophobicity. Pang et al. (1998) compared the breakthrough of a dye tracer and *B. subtilis* spores in an alluvial gravel aquifer. The retention of the spores was attributed to filtration, sedimentation, and irreversible absorption. A similar experiment was conducted by Sinton et al. (2000) and the authors came to the same conclusions.

Systematic studies comparing the transport behavior of oocysts and spores over a range of physicochemical conditions have not yet been performed. However, available literature provides valuable insight on expected trends. A first-order kinetic expression is frequently employed to account for microbial retention. Filtration theory (Yao et al., 1971) attempts to predict the retention rate coefficient (k_{sw}, T^{-1} where T denotes units of time) as:

$$k_{sw} = \frac{3(1-\theta_w)}{2d_{50}}\alpha\eta v \qquad [1]$$

where v [LT^{-1}] is the average pore water velocity, d_{50} [L] is the median grain diameter, η [-] is the single collector contact efficiency, and α [-] is the sticking efficiency. Mass transfer of microbes to the porous medium surface is quantified by η. Correlation equations to predict η as a function of system variables (water velocity, microbe size and density, and grain size) have been developed from pore-scale simulations of microbe transport in simplified grain geometries under water saturated conditions (e.g., Rajagopalan and Tien, 1976; Tufenkji and Elimelech, 2004a; Nelson and Ginn, 2005; Ma et al., 2009).

All collisions with the grain surfaces lead to microbe immobilization when $\alpha = 1$. In this case, differences in k_{sw} reflect the influence of only mass transfer. Fig. 5a and b presents predicted values of k_{sw} for spores and oocysts as a function of Darcy water velocity (q_w, LT^{-1} where L denotes units of length) and median grain diameter (d_{50}, L), respectively, when $\alpha = 1$. These predictions were obtained using Eq. [1] in conjunction with η determined using the correlation equation of Tufenkji and Elimelech (2004). The spore and oocyst were assumed to have a density of 1.3 and 1.045 g cm^3, and a size of 2 and 6 μm (upper reported size limits), respectively. Observe that differences in k_{sw} for spores and oocysts mainly occur at larger velocities ($q_w > 0.1$ cm min^{-1}) and for smaller grain sizes ($d_{50} < 250$ μm). In both of these cases the value of k_{sw} is larger for oocysts than spores. This same trend also held when the using the lower size limits for spores (1 μm) and oocysts (3.5 μm) (data not shown). Hence, mass transfer considerations indicate that spores should provide a conservative estimate of oocyst transport because spores exhibit less retention for the same value of α.

In reality, the value of α is commonly much less than 1 and only

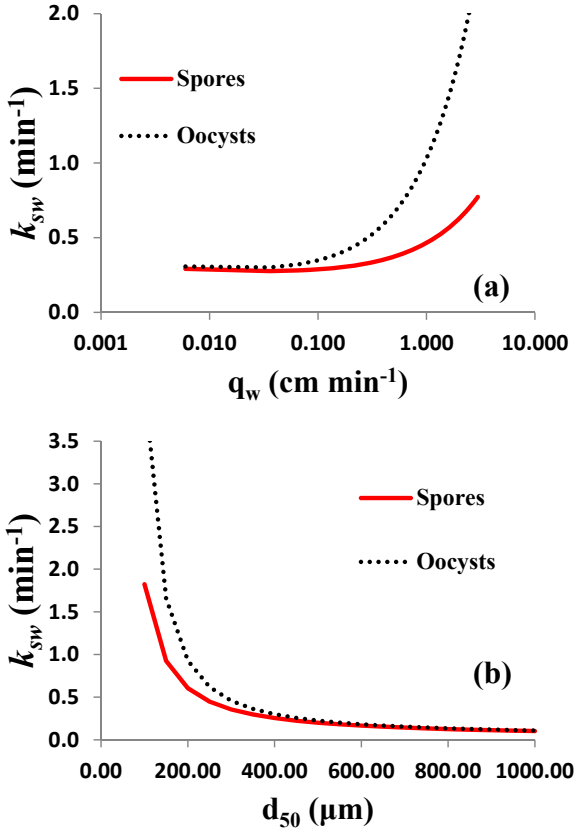

Fig. 5. Predicted values of k_{sw} for spores and oocysts as a function of q_w (a) and d_{50} (b), respectively, when $\alpha = 1$. These predictions were obtained using Eq. [1] in conjunction with η determined using the correlation equation of Tufenkji and Elimelech (2004a,b). The spore and oocyst were assumed to have a density of 1.3 and 1.045 g cm^3, and a size of 2 and 6 μm (upper reported size limits), respectively.

a fraction of the solid surface contributes to retention (S_f). The values of α and S_f are determined by the forces and torques (arising from adhesion, hydrodynamics, and Brownian diffusion) that act on a microbe adjacent to the grain surface (Cushing and Lawler, 1998; Bradford et al., 2013; Bradford and Torkzaban, 2012, 2015). Consequently, experimental values α and S_f for microbes and colloids are sensitive to the solution chemistry (Tufenkji and Elimelech, 2005a; Shen et al., 2007), the surface properties of the grains and microbe (Kim et al., 2009, 2010a, 2010b; Bolster et al., 2009, 2010; Foppen et al., 2010), and the system hydrodynamics (Johnson et al., 2007; Torkzaban et al., 2007; Shen et al., 2010; Sasidharan et al., 2014). Theoretical calculations confirm these findings, and also indicate that α and S_f will be functions of the microbe deformation, size, and surface charge, the grain size, the amount and size of the chemical heterogeneity and nanoscale roughness, and microscale roughness (Bradford and Torkzaban, 2012, 2015; Bradford and Torkzaban, 2013b). In general, surface straining at microscopic roughness locations increases with microbe size and water velocity, and decreasing ionic strength (Bradford and Torkzaban, 2015). Conversely, attachment increases with ionic strength and chemical heterogeneity, and for deceasing microbe size, water velocity, and density of nanoscale roughness (Bradford and Torkzaban, 2015). Due to all of these complexities the values of α and S_f are frequently viewed as empirical parameters that are obtained by fitting to measured transport data (Tufenkji and Elimelech, 2005a; Bradford et al., 2014). These observations point to the need for experimental values of α and S_f over a range of physicochemical conditions to assess the ability of spores to serve as a surrogate for oocyst transport behavior.

In addition, the value of k_{sw} may be further decreased over time as available retention sites become blocked or filled. Blocking causes microbial and colloid breakthrough concentrations to increase with time and retention profiles to approach a constant value with depth (Tan et al., 1994; Bradford et al., 2014). Retarded breakthrough curves may also occur as a result of blocking if the value of k_{sw} is sufficiently large (Harmand et al., 1996; Schijven and Hassanizadeh, 2000). Note that blocking will be highly sensitive to the input pulse duration and concentration when S_f is small (Bradford et al., 2009; Sasidharan et al., 2014). Small values of S_f (<2%) commonly occur in porous media, especially for larger microbes, even under chemical conditions that are predicted to be favorable for microbe retention (Truemann et al., 2014; Sasidharan et al., 2014). Additional complications arise when the microbial suspension is aggregating. Changes in the size distribution will influence rates of microbial mass transfer to the grain surface (Tufenkji and Elimelech, 2004), and adhesive and hydrodynamic interactions (Bradford and Torkzaban, 2015). Furthermore, retained microbes can act as favorable locations for subsequent retention, a process known as ripening (Liu et al., 2008; Tong et al., 2008). Ripening will increase the value of k_{sw} and decrease breakthrough concentrations over time (Bradford et al., 2014). These observations indicate that differences in the transport behavior of oocysts and spores may occur over time, and that long-term transport monitoring may be warranted under certain instances (e.g., at high input concentration levels and long seeding times).

8.3. Release

Persistent, low levels of oocyst release have been observed under steady-state physicochemical conditions (Harter et al., 2000; Bradford and Bettahar, 2005; Cortis et al., 2006; Kim et al., 2010b). In this case, microbial release is a diffusion controlled process (Ryan and Gschwend, 1994; Ryan and Elimelech, 1996). Specifically, if the depth of the interaction energy minimum is sufficiently small then random fluctuations in the kinetic energy of diffusing microbes allow for periodic escape from the minimum and subsequent transport away from the grain surface. Hence, the rate of oocyst release has been observed to decrease with increasing ionic strength (Cortis et al., 2006) because the depth of the minimum in the interaction energy increases.

Harter et al. (2000) found that up to half of the initial attachment of oocysts appeared to be reversible. In addition, multiple sites for kinetic retention and release have been required to describe oocyst transport (Bradford and Bettahar, 2005; Tufenkji and Elimelech, 2005a). These observations partially reflect differences in the strength in the interaction energy and/or hydrodynamics conditions on the surface of the porous medium. In particular, spatial variations in primary minimum interactions can arise under unfavorable attachment conditions as a result of localized nanoscale roughness and/or chemical heterogeneity (Hoek et al., 2003; Duffadar and Davis, 2008; Huang et al., 2010; Bendersky and Davis, 2011). Note that primary minimum interactions are typically stronger and less reversible than secondary minimum interactions (Shen et al., 2012; Bradford and Torkzaban, 2013). Furthermore, variations in the pore space geometry, grain size distribution, and microbe size in a representative elementary volume of a porous medium will produce a distribution of fluid drag forces that act on a microbe at the solid–water interface (Bradford et al., 2011). Hydrodynamic removal of microbes from microscopic roughness locations and grain–grain contracts will be less likely than on a smooth surface because of lower hydrodynamic forces, eddy zones, and alteration of the lever arms associated with adhesive and hydrodynamics torques (Torkzaban et al., 2008;

Bradford and Torkzaban, 2013b). In general, the influence of nanoscale chemical heterogeneity is diminished and hydrodynamic forces are greater for larger microbes (Bradford and Torkzaban, 2012; Bradford et al., 2015). Consequently, larger oocysts are anticipated to be more susceptible to diffusive and hydrodynamic removal than spores. In addition, it makes no biological sense for an oocyst to irreversibly interact with a soil particle when mammalian epithelial cells are the target. Evolutionary process would have ensured that a mechanism exists to deliver the oocysts to the host.

Transient physicochemical conditions may occur in the subsurface as a result of infiltration and recharge, groundwater and surface water interactions, injection and/or extraction wells, and contamination events. Significant amounts of oocyst release have been observed to rapidly occur with a decrease in solution ionic strength and/or an increase in pH (Tufenkji and Elimelech, 2005a; Kim et al., 2010b). Transients in water velocity have also been shown to produce release of oocysts (Kim et al., 2010b) and bacteria (Bradford et al., 2015). A reduction in adhesive torque (decreasing ionic strength or increasing pH) and/or an increase in the hydrodynamic torque (water velocity) produces microbe release when the applied hydrodynamic torque becomes greater than the resisting adhesive torque (Bergendahl and Grasso, 1998, 1999). However, microscopic roughness and grain–grain contacts may shield microbes from the effects of increasing velocity because of their influence on lever arms (e.g., the lever arm for the applied hydrodynamic torque is zero when the roughness height is greater than the microbe radius). In this case, oocyst release can actually be enhanced with a flow interruption (Kim et al., 2010b) that allows oocysts to diffuse away from these locations. The relative influence of transients in physicochemical conditions on oocyst and spore release has not yet been determined.

9. Survival

Oocysts are very resistant to chemical challenges (Table 1), particularly chlorination during water treatment (Korich et al., 1990; Carmena et al., 2007), and can remain infective for months while lying dormant in the environment (Robertson et al., 1992; Harris and Petry, 1999), but they are very susceptible to extremes in temperature (King and Monis, 2007). At low temperatures (~<15 °C) oocysts can survive for long periods, but at high temperatures (>35 °C) they can be killed (Fayer et al., 1998; Davies et al., 2005; King and Monis, 2007). Temperatures above 55 °C inactivate oocysts (Fujino et al., 2002), possibly due to melting of lipids in the central portion of the wall (Jenkins et al., 2010). Freezing can kill oocysts as well, but some oocysts can remain infective to temperatures of at least −22 °C (Robertson et al., 1992; Fayer et al., 1998). Desiccation is completely lethal to oocysts (Robertson et al., 1992) and oocysts are also sensitive to UV radiation and do not repair UV induced DNA damage (Shin et al., 2001). However, in the soil, many oocysts can likely find protection from these environmental factors and maintain their infectivity long enough to encounter a host (King and Monis, 2007).

Similar to oocysts, aerobic spores are resistant to many chemicals (Table 1) and have elevated (though not complete) resistance to chlorination (Russell et al., 1990; Barbeau et al., 1999; Rice et al., 2005). But unlike oocysts, spores are resistant to extreme environmental conditions such as heat, desiccation, and radiation common to many natural environments (Setlow, 2006; Leggett et al., 2012). As noted previously, the water content in the core is low and this makes the spore more resistant to heat and desiccation (Bearman et al., 1982; Setlow, 2006; Sunde et al., 2009). Studies have shown that spores with high calcium concentrations are the most resistant to heat, while cores without DPA are more hydrated (Paidhungat et al., 2000; Setlow, 2006). Spores can withstand temperatures over 100 °C in both wet and dry heat and low temperatures near −80 °C (Nicholson et al., 2000). Spores are also structurally flexible and can respond to environmental changes such as changes in humidity levels (Henriques and Moran, 2007; Driks, 2009; Westphal et al., 2003). Spores contain no energy reserves and cannot repair any DNA damage while dormant (Setlow, 2006), although some spores have been found to possess UV absorbing pigments in their outer layers (Nicholson et al., 2005 and references within).

The advantages of utilizing a surrogate for oocysts that is dormant should not be overlooked. Vegetative surrogates add additional variables to transport studies that complicate experiments (e.g., growth, death, and chemotaxis). In addition to viability issues, vegetative surrogates can react to experimental conditions (particularly solution chemistry) in a manner that is not reflective of a dormant biology. Bacteria are known to chemically alter their environment, especially in nature (e.g., Johnson and Hallberg, 2003; McIntosh et al., 2004). Aside from responding to possible germinants (which can be minimized in laboratory studies), spores will remain dormant across a wide range of solution chemistries and experimental conditions that investigators may wish to examine. Spores can also survive in any environment oocysts can survive in and many extreme environments where oocysts will not remain viable, which make them an excellent (conservative) surrogate.

10. Prevalence in the environment

Cryptosporidium oocysts are generally only found in low concentrations in surface waters (Cornwell et al., 2003; Guy et al., 2003; Hörman et al., 2004; Briancesco and Bonadonna, 2005; Karanis et al., 2006; Brown and Cornwell, 2007); however their prevalence in surface waters and soils near animal operations is much higher (Hansen and Ongerth, 1991; Bodley-Tickell et al., 2002; Heitman et al., 2002; Sturdee et al., 2003; Dorner et al., 2004) (Table 1). In addition, oocyst production in animals tends to be seasonal (Sturdee et al., 2003; Dorner et al., 2004; Wilkes et al., 2009). This is not surprising since oocyst infection is dependent on the availability of a suitable mammalian host. While oocysts can be transported considerable distances, oocyst concentrations would be expected to be highest when and where suitable hosts are present. The high infectivity of oocysts coupled with the seasonality can make monitoring of waters for oocysts more challenging. Even when oocysts concentrations are low it still only takes a small number of oocysts to cause infection.

On the other hand, aerobic spores are ubiquitous in the environment (Nicholson et al., 2000) although some aerobic spores have been found to exhibit seasonality, particularly in dairy operations (Kauri, 1982; Phillips and Griffiths, 1986; Slaghuis et al., 1997; Buehner et al., 2014) (Table 1). Aerobic spores are also present in much greater concentrations in the environment than other proposed surrogates for oocysts such as anaerobic spores (Weiss et al., 2005). Aerobic bacterial spores are not (necessarily) dependent on a host and can be produced by sporulating bacteria at anytime and anywhere in the environment where nutrient limitations occur. The high prevalence of aerobic spores in the environment make them much easier to detect and monitor in water sources.

In 2012, the EPA published the Unregulated Contaminant Monitoring Rule 3 (UCMR3) which lists 28 unregulated chemical contaminants and 11 microbial contaminants that are to be monitored by public water systems. Between January 2013 and May 2015, Public Water Systems (PWS) from 21 states reported the results of microbial monitoring. Microbial contaminants were reported in PWS from 16 of these states and aerobic spores were reported in more of the PWS than the other ten microbial

contaminants combined (U.S. EPA, 2015). Aerobic spores were reported in ~28% of the PWS monitoring for microbial contaminants, while only 4.5% reported the presence of total coliforms, 5% reported the presence of Enterococci, and less than 1% reported the presence of *E. coli* (Table 2) (U.S. EPA, 2015). Surrogates that are difficult to detect in the environment because of low concentrations or survivability issues (like many fecal indicators) are unlikely to be useful surrogates in the long term. The ubiquitous nature of aerobic spores indicates that they are capable of being readily transported in the environment and they are present at concentrations that make them easily detected by PWS. Furthermore, aerobic spores are more likely to survive water treatment processes than other proposed surrogates making them an ideal conservative surrogate for oocysts.

11. Conclusions

The case for aerobic bacterial spores serving as a surrogate for cryptosporidium oocysts to determine surface water contamination and GWUDI of surface water is strong. The biology and survivability of aerobic spores are strikingly similar to oocysts. Furthermore, the prevalence and ease of detection of aerobic spores

Table 2
Microbiological contaminant monitoring results (U.S. EPA, 2015).

Contaminant	MRL[a]	Unit	Total number of PWS with results	Total number of PWS with ≥MRL
Aerobic Spores	1	SFO/100 ml	710	198
E. coli	1	MPN/100 ml	708	3
Enterococci	1	MPN/100 ml	707	37
Enterovirus (cell culture)	0.002	MPN/L	706	2
Enterovirus (RT-qPCR)	0.398	GC/L	706	6
Male specific phage	1	PFU/100 ml	692	10
Notovirus GIA	0.398	GC/L	706	0
Notovirus GIB	0.398	GC/L	706	1
Notovirus GII	0.398	GC/L	706	4
Somatic phage	1	PFU/100 ml	692	3
Total Coliforms	1	MPN/100 ml	708	32

SFO = Spore Forming Units, MPN = Most Probable Number, GC = Genomic Copies, PFU = Plaque forming Units.
[a] Minimum Reporting Level. The MRL were established based on the capability of the analytical method.

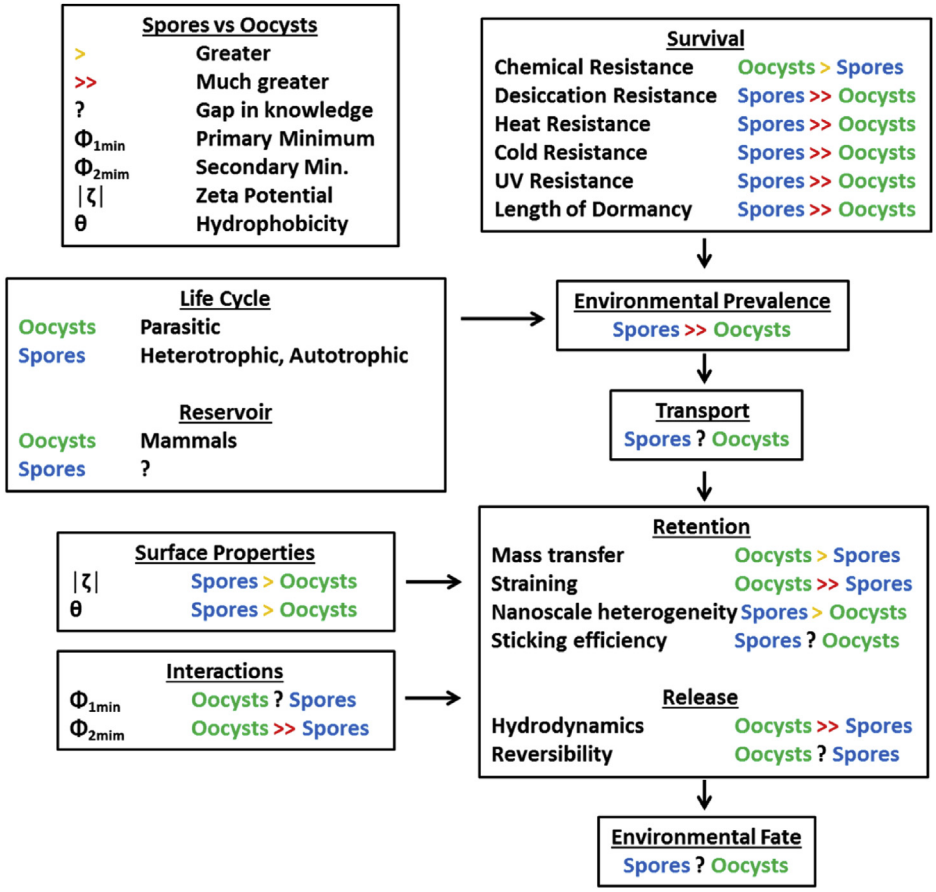

Fig. 6. A schematic comparing processes (e.g., survival, environmental prevalence, transport, retention, and release) that influence the environmental fate of aerobic spores and cryptosporidium oocysts. Note that environmental prevalence is influenced by the microbe life cycle, reservoir, and survival, whereas retention and release are influenced by surface properties and interactions. Gaps in knowledge are identified with question marks.

makes them a practical and cost-effective solution for use in both laboratory studies and in water treatment monitoring. However, there are some issues that need to be addressed to ensure that aerobic spores are a conservative surrogate for the fate oocysts in the subsurface environment.

Fig. 6 presents a schematic illustrating the main conclusions of this review with regard to what is currently known about processes (e.g., survival, environmental prevalence, transport, retention, and release) that influence the environmental fate of aerobic spores and cryptosporidium oocysts. Note that environmental prevalence is influenced by the microbe life cycle, reservoir, and survival, whereas retention and release are influenced by surface properties and interactions. Gaps in knowledge are identified with question marks.

A wide variety of surface charge and hydrophobicity properties have been reported for aerobic spore species. This variability will undoubtedly produce a diversity of surface interactions and transport behavior in the environment. Hence, not all aerobic spores are expected to be suitable surrogates for oocyst migration in laboratory and monitoring studies. A systematic effort needs to be made by researchers to identify which aerobic spores are (and are not) the best surrogate. There is no reason to expect that a single aerobic spore will be the answer. Indeed, it is likely that multiple aerobic spores will prove to be adequate surrogates and some may be suitable only for specific objectives. Selection of the aerobic spore species will require careful consideration of purification procedures, surface charge, surface hydrophobicity and measurement methodology, and transport behavior. We strongly advocate an approach that involves making direct comparisons between oocysts and potential surrogates in laboratory studies to obtain strong quantitative data to make good selections. Admittedly, this is not a trivial task, but simply using the most easily obtained aerobic spores or selecting based on literature review may prove to be counterproductive in the long run.

The long term transport and release of aerobic spores in the environment needs to be examined more thoroughly and compared to the work that has been done with oocysts over a range in physicochemical conditions. Spore and oocyst mass transfer coefficients indicate that spores will provide a conservative estimate of oocysts retention when the sticking efficiencies and reversibility are similar. Furthermore, oocysts are much more susceptible to straining than aerobic spores because of their larger size. Possible differences in the release behavior of oocysts and spores are of special concern, because only of fraction of the oocysts are irreversibly retained and significant amounts of oocyst release have been observed under steady-state and transient conditions. Another area that needs further examination is the fate of aerobic spores in the environment, in particular when and where they germinate in the environment. The assumption is that the life cycle of aerobic spores is cyclical like that of oocysts, but this may not always be the case and further research in this area would be helpful to more fully understand the fate of spores in natural environments.

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Appendix A. Supplementary data

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