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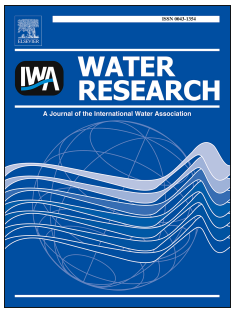

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PII: S0043-1354(16)30690-X

DOI: 10.1016/j.watres.2016.09.013

Reference: WR 12352

To appear in: *Water Research*

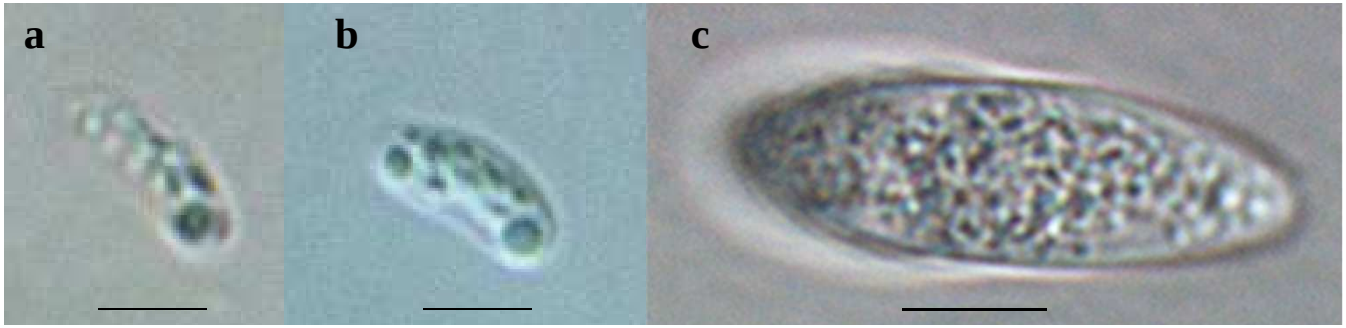
Received Date: 5 May 2016

Revised Date: 7 September 2016

Accepted Date: 8 September 2016

Please cite this article as: Ryan, U., Paparini, A., Monis, P., Hijjawi, N., It's official – *Cryptosporidium* is a gregarine: What are the implications for the water industry?, *Water Research* (2016), doi: 10.1016/j.watres.2016.09.013.

Novel *Cryptosporidium* life cycle stages



Review

It's official – *Cryptosporidium* is a gregarine: what are the implications for the water industry?

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Abstract

Parasites of the genus *Cryptosporidium* are a major cause of diarrhoea and ill-health in humans and animals and are frequent causes of waterborne outbreaks. Until recently, it was thought that *Cryptosporidium* was an obligate intracellular parasite that only replicated within a suitable host, and that faecally shed oocysts could survive in the environment but could not multiply. In light of extensive biological and molecular data, including the ability of *Cryptosporidium* to complete its life cycle in the absence of a host and the production of novel extracellular stages, *Cryptosporidium* has been formally transferred from the Coccidia, to a new subclass, Cryptogregaria, with gregarine parasites. In this review, we discuss the close relationship between *Cryptosporidium* and gregarines and discuss the implications for the water industry.

Keywords: *Cryptosporidium*; gregarine; cell-free; gamont-like extracellular stages; water industry;

1 Introduction

The Apicomplexan parasite *Cryptosporidium* is a major cause of severe diarrhoea, developmental problems and death in young children and chronic, life-threatening disease in immunocompromised and malnourished individuals (Guerrant et al., 1999; Snelling et al., 2007; Costa et al., 2011; Kotloff et al., 2013; Striepen, 2013). No vaccines are available for *Cryptosporidium* (Mead, 2014) and current treatment options for cryptosporidiosis are limited, with only one drug, nitazoxanide (NTZ), exhibiting moderate clinical efficacy in children and immunocompetent people, and none in people with HIV (Abubakar et al., 2007; Amadi et al., 2009). Of the 31 valid species (Costa et al., 2016; Li et al., 2015; Ryan et al., 2015; Holubová et al., 2016; Kváč et al., 2016; Zahedi et al., 2016), *Cryptosporidium parvum* and *Cryptosporidium hominis* are responsible for the majority of human infections, although in some countries, *C. meleagridis* is as prevalent as *C. parvum* in human populations (Xiao, 2010).

Transmission of the parasite occurs via the faecal-oral route, either by ingestion of contaminated water or food, or by human-to-human or animal-to-human transmission (Xiao, 2010). The World Health Organization has categorized *Cryptosporidium* as a reference pathogen for the assessment of drinking water quality (Medema et al., 2006). This is because oocysts produced by *Cryptosporidium* are extremely hardy, easily spread via water, resistant to inactivation by chlorine and are difficult to remove from drinking water, without the use of expensive and lengthy filtration (Jakubowski, 1995; Striepen and Kissinger, 2004).

Waterborne transmission is a major mode of transmission and *Cryptosporidium* was the etiological agent in 60.3% (120) of the waterborne protozoan parasitic outbreaks that have been reported worldwide between 2004 and 2010 (Baldursson and Karanis, 2011). The severity of infections vary, depending on the species involved, but for zoonotic species, the dose required to cause an infection in 50% of subjects (ID 50) is estimated to be 10–83 oocysts for *C. hominis* and

132 for *C. parvum* (DuPont et al., 1995; Okhuysen et al., 1998; Chappell et al., 2006). The minimum infectious dose for *C. meleagridis* has yet to be determined (Chappell et al., 2011). Although the lowest infectious dose for *C. hominis* has been calculated to be 10 oocysts, in reality, one oocyst could be sufficient to cause infection in humans through direct or indirect routes of transmission (Chappell et al., 2006).

In addition to the apical complex, one main and unique feature of the phylum Apicomplexa, to which *Cryptosporidium* belongs, is the widespread presence of the apicoplast. This four-membrane-encased relict plastid (35kb genome) of secondary endosymbiotic origin is thought to have originated by engulfment of a chloroplast-containing alga by the primitive eukaryotic ancestor of the Apicomplexa (Lim and McFadden, 2010). Microscopic, molecular, genomic and biochemical data indicate that *Cryptosporidium* differs from other apicomplexans in that it has lost the apicoplast (like the colpodellids and other gregarines) (rev. in Lim and McFadden, 2010), as well as the genomes for both the plastid and the mitochondrion (Zhu et al., 2000; Abrahamsen et al., 2004; Xu et al., 2004). *Cryptosporidium* also differs from other apicomplexans in fundamental features such as motility and invasion (Wetzel et al., 2005).

Until recently, *Cryptosporidium* was classified as a coccidian parasite. However, it has long been speculated that *Cryptosporidium* represents a 'missing link' between the more primitive gregarine parasites and coccidians. The similarities between *Cryptosporidium* and gregarines have been supported by extensive microscopic, molecular, genomic and biochemical data (Pohlenz et al., 1978; Bull et al., 1998; Carreno et al., 1999; Beyer et al., 2000; Hijjawi et al., 2002; Leander et al., 2003a; Hijjawi et al., 2004; Rosales et al., 2005; Barta and Thompson, 2006; Butaeva et al., 2006; Valigurová et al., 2007; Boxell et al., 2008; Karanis et al., 2008; Zhang et al., 2009; Borowski et al., 2008; 2010; Hijjawi, 2010; Hijjawi et al., 2010; Templeton et al., 2010; Karanis and Aldeyarbi, 2011; Boxell, 2012; Koh et al., 2013, 2014; Huang et al., 2014; 2015; Clode et al., 2015; Valigurová et al., 2015; Aldeyarbi and Karanis, 2016a; 2016b; 2016c; Edwinson et al., 2016;

Paziewska-Harris et al., 2016), which have served as the basis for the formal transfer of *Cryptosporidium* from subclass Coccidia, class Coccidiomorphea to a new subclass, Cryptogregaria, within class Gregarinomorphea (Cavalier-Smith, 2014). The genus *Cryptosporidium* is currently the sole member of Cryptogregaria and is described as comprising epicellular parasites of vertebrates possessing a gregarine-like feeder organelle but lacking an apicoplast (Cavalier-Smith, 2014). According to the International Code of Zoological Nomenclature (ICZN) (http://www.iczn.org/iczn/index.jsp), once a species has been formally re-classified in a peer-reviewed publically available journal, then that re-classification stands (unless challenged in the literature). As this re-classification has not been challenged, *Cryptosporidium* is now officially a gregarine.

2.0 What are gregarines?

Gregarines (phylum Apicomplexa; class Gregarinomorphea) are a very diverse group of large, single-celled "primitive" apicomplexan parasites that primarily infect the intestines and other extracellular spaces of invertebrates and lower vertebrates (mainly arthropods, molluscs and annelids), which are abundant in natural water sources (Leander et al., 2003a; 2003b; Barta and Thompson, 2006; Leander, 2007; Valigurová et al., 2007). The transmission of gregarines to new hosts usually takes place by oral ingestion of oocysts in both aquatic and terrestrial environments. Four or more sporozoites (depending on the species) escape from the oocysts, find their way to the appropriate body cavity and attach to, or penetrate, the host cells. The sporozoites emerge from a host cell, begin to feed and develop into large trophozoites (Rueckert and Leander, 2008).

Many gregarines do not exhibit intracellular stages and are mostly epicellular parasites. The gregarine life cycle typically only consists of gametogony and sporogony and only a few species exhibit merogony. The sporozoites will generally develop into large trophozoites and attach to the

host cell with a specialised attachment apparatus (epimerite, mucron, modified protomerite) (MacMillan, 1973). These specialized structures are derived from the conoid at the apical end. This attachment to the host cell also functions in feeding in that the cytoplasm of the host is taken up by the attached parasite (i.e., myzocytosis) (Valigurová et al., 2007). Two mature trophozoites eventually pair up in a process called syzygy and develop into gamonts. The orientation of gamonts during syzygy differs depending on the species (e.g. side-to-side and head-to-tail). A gametocyst wall forms around each pair of gamonts, which then begins to divide into hundreds of gametes (gametogeny). Pairs of gametes fuse and form zygotes, each of which becomes surrounded by an oocyst wall. Within the oocyst, meiosis occurs to yield four or more spindle-shaped sporozoites (sporogony). Hundreds of oocysts accumulate within each gametocyst, and are usually released via host faeces or via host death and decay (Vivier and Desportes, 1990; Kuriyama et al., 2005; Rueckert and Leander, 2008).

The gregarines are thought to be the earliest lineage of apicomplexans (Rueckert and Leander, 2008) and were previously subdivided into three orders; Archigregarinida, Eugregarinida and Neogregarinida (Adl et al., 2012; Grassé 1953). However, the taxonomy has recently been revised (Cavalier-Smith, 2014), on the basis that it was phylogenetically unsound (Rueckert et al., 2011). In this new classification, the class name Gregarinomorphea has been adopted to broadly refer to all its members (i.e. gregarines, *Cryptosporidium* and Histogregaria) (Cavalier-Smith, 2014). Within the various subclasses of Gregarinomorphea are Cryptogregaria, discussed above, and Orthogregarinia (comprising the orders Vermigregarida and Arthrogregarida), for gregarines most closely related to *Cryptosporidium* (Cavalier-Smith, 2014).

3. Key similarities between gregarines and *Cryptosporidium*

Similarities between *Cryptosporidium* and gregarine parasites are outlined in Table 1. Key similarities include the ability of *Cryptosporidium* to complete its life cycle in the absence of a host, the presence of large extracellular gamont stages, syzygy (end to end pairing for reproduction) and ability to adapt to their environment by changing their cell structure depending on the surrounding environment.

3.1 Ability to complete its life cycle in the absence of host cells

Until recently, it was thought that *Cryptosporidium* were obligate intracellular parasites that completed their life-cycle in an intra-cellular but extra-cytoplasmic (epicellular) location by pulling the host cell membrane around it as an extracytoplasmic "parasitophorous sac/ membrane" that sequestrated the parasite from the intestinal lumen and the host cell's cytoplasm (Tzipori and Ward, 2002; Dumenil, 2011). However, the initial description of the complete development of *Cryptosporidium* in axenic culture (without attachment to host cells) by Hijjawi et al. in 2004, revealed that *Cryptosporidium* is not an obligate epicellular parasite and this has been confirmed by subsequent studies (Boxell et al., 2008; Hijjawi et al., 2010; Boxell, 2012; Yang et al., 2015; Aldeyarbi and Karanis, 2016a; 2016b; 2016c). Studies by Aldeyarbi and Karanis have confirmed the presence of all known life cycle stages and the production of both thin and thick-walled oocysts by transmission electron microscopy (TEM) (Aldeyarbi and Karanis, 2016b; 2016c). Even when *Cryptosporidium* is cultivated with host cells, it has been reported that as *C. parvum* progresses through its life cycle, it becomes more extracellular with no evidence of attachment to cell lines found (Borowski et al., 2010). Another study on quantitative PCR (qPCR) analysis of *Cryptosporidium* growth in both cell culture and cell-free culture, reported that only ~ 5% of parasite DNA could be found associated with host cells or bound to the plastic of the cell-free cultures, and that the majority of parasite DNA was present in the cell culture medium (Paziewska-

Harris et al., 2016). These findings support the earlier observations by Pohlenz et al. (1978) and Beyer et al. (2000), where intact various developing stages of parasites that are not enclosed within parasitophorous sacs were found free in the calves' lumens or deep within free macrophages. This is despite the fact that the very process of taking sections of intestine and processing for histology analysis is likely to wash away anything not directly attached to enterocytes.

The ability of *Cryptosporidium* to complete its life cycle extracellularly also further confirms its relationship with gregarines. For example, coelomic gregarines can also survive extracellularly, even without attaching to the host intestine (Desportes and Schrével, 2013). It therefore appears that *Cryptosporidium* is capable of both epicellular and extracellular multiplication and development and they may both occur simultaneously in the host for mass production of new oocysts (Clode et al., 2015). Preliminary work also suggests that *Cryptosporidium* can complete its life cycle in water (Boxell, 2013), although this needs further validation.

3.2 Extracellular gamont-like stages

The presence of gamont-like extracellular stages in the life cycle of *Cryptosporidium* was first observed in a study by Hijjawi et al. (2002) and has since been reported by several investigators (Hijjawi et al., 2004; Rosales et al., 2005; Karanis et al., 2008; Borowski et al., 2010; Koh et al., 2013, 2014; Huang et al., 2014; Aldeyarbi and Karanis, 2016a). A previous study had suggested that the presence of gamont-like stages in both cell-free and in-vitro cultures was due to contaminating debris or fungal infection resembling *Bipolaris australiensis* and *Colletotrichum acutatum* (Woods and Upton, 2007). However, TEM analysis of gamont stages (Aldeyarbi and Karanis, 2016a), counters this argument.

Extracellular gamont-like stages have been purified from cell-free culture and *in vivo* from mice infected with *C. parvum* (Hijjawi et al., 2004, Fig. 1a). Pairing of these gamont-like stages with each other in a process similar to syzygy (Fig 1b), resulted in the formation of a gametocyst (multi-nucleated mass)-like stage, which originated after their fusion (Fig. 1c). The identity and the role of these stages are still unknown but similar cell sizes and morphologies have been observed in gregarines (Leander, 2006, Leander, 2007; Alarcón et al., 2011). It has been suggested that gamont-like extracellular stages might originate from sporozoites which failed to penetrate the host cells and developed extracellularly into motile trophozoite stages (Hijjawi et al., 2004; Rosales et al., 2005). However, given the dominance of the trophozoite stage in the life cycle (Hijjawi et al., 2004; Borrowski et al., 2010; Yang et al., 2015), it is possible that they are derived from trophozoite stages. Interestingly, trophozoites and developing meronts showing dividing nuclei have been observed inside of unexcysted oocysts (Borrowski et al., 2010; Hijjawi et al., 2010; Aldeyarbi and Karanis, (2016b) and in certain instances, *Cryptosporidium* sporozoites /zoites have the ability to develop directly to sexual stages during in vitro cell-free culturing without appearing to go through a merogenic process (Aldeyarbi and Karanis, 2016c). This plasticity in its life cycle, with the ability to avoid merogony and initiate mitotic division from fused sporozoites is similar to Urosporoidea (formerly eugregarines) (Rueckert et al., 2013). It is possible that gametogenesis may occur inside the gametocyst-like stage and that mature oocysts are released in clumps upon its disintegration. This could explain why oocysts are often seen clumped together in faecal and water samples (Hijjawi, unpublished observations). Further research is required to better understand this process.

3.3 Syzygy

A defining characteristic of gregarines is syzygy (the process in which two mature trophozoites pair up before the formation of a gametocyst) (Rueckert and Leander, 2008). For

Cryptosporidium, the process of syzygy (end to end pairing for reproduction) was first described by Hijjawi et al. (2002). In that study, large (~10 µm) extracellular stages of *C. andersoni*, present in large numbers in the faeces of infected cattle, were observed undergoing syzgy. Isolation of this stage using laser microdissection and subsequent molecular characterisation confirmed that this was a stage in the life cycle of *C. andersoni* (Hijjawi et al., 2002). Stages similar to these have been described in the gregarine *Heliospora caprellae* (Rueckert et al., 2011). Since then, pairing of *Cryptosporidium* merozoites type II/I (Borowski et al., 2010), extracellular trophozoite/gamont associations (Rosales et al. 2005; Koh et al. 2014), lateral pairing between trophozoites or sporozoites (Hijjawi et al., 2004; Hijjawi et al. 2010) and latero-caudal or side-by-side syzygy of extracellular stages or gamonts (Aldeyarbi and Karanis, 2016a) and pairing of extracellular microgametes (Aldeyarbi and Karanis, 2016c) has been reported. The latter studies by Aldeyarbi and Karanis, (2016c) reported that pairing of extracellular microgametes resembles the caudo-caudal syzygy of the archigregarines *Selenidiidae Selenidium pendula*, *Selenidium hollandei* (Desportes and Schrével, 2013) and *Selenidium pennatum* (Kuvardina and Simdyanov, 2002). The exact identity of such pairing in *Cryptosporidium* remains unknown, but it has been suggested that this could be due to affinity between *Cryptosporidium* stages/gamonts rather than biological purposes as in gregarines (Aldeyarbi and Karanis, 2016c).

3.4 Ability to adapt to their environment (variation in cell structure feeding modes)

Gregarines exhibit an enormous diversity in cell architecture and dimensions, depending on their parasitic strategy and the surrounding environment (Leander et al. 2003b; Leander 2008; Valigurová, 2012), which is also reflected in variation in feeding modes (epimerite, mucron, modified protomerite) (MacMillan, 1973). This ability to adapt to their environment is also seen with *Cryptosporidium*, which also appears to exhibit tremendous variety in cell structure depending

on the surrounding environment (Aldeyarbi and Karanis, 2016c). For example, the extension of the pellicle in microgamonts may play a role the parasite's adjustment for nutrient acquisition through increasing its surface area, as reported in other cell-free *Cryptosporidium* asexual and gregarine-like stages (Aldeyarbi and Karanis 2016a, 2016b, 2016c). These adaptions in *Cryptosporidium* may have developed to allow it to survive and grow in cell-free conditions.

4. What does this mean for the water industry?

The recent breakthroughs in *Cryptosporidium* biology (i.e., reclassification and confirmed ability for host-free replication) could represent a paradigm shift for the water industry, particularly in the area of distribution system risk. Should *Cryptosporidium* be able to access the energy available in bacterial biofilms and multiply in (drinking) water distribution systems, the current understanding of the impact of this pathogen and its risk assessment would need to be revised.

4.1 Do current anti-Cryptosporidium antibodies cross react with novel gamont-like stages?

Currently two types of antibodies are used in the water industry: (1) oocyst-specific antibodies that react with the oocyst wall (various suppliers) and (2) Sporo-Glo (Waterborne Inc.) which is specific for sporozoites and other intracellular life cycles stages. The latter specifically targets *Cryptosporidium* developmental stages by targeting antigens exposed only after excystation (Boxell et al., 2008; Edwards et al., 2012; Koh et al., 2013). Non-commercial antibodies have also been raised against oocysts and life-cycle stages including an antibody (anti-Cp33) that recognizes a 33 kDa protein on *C. parvum* but not *C. hominis* sporozoites (Jenkins et al., 2014).

As thick-walled *Cryptosporidium* oocysts are the infectious stage and the life form currently believed to be the only stage in the life cycle capable of surviving in the environment, current

257 detection methods for *Cryptosporidium* in water have focused on detecting oocysts using the US
258 EPA 1623 method, utilising various fluorescent *Cryptosporidium* oocyst wall antibodies (DiGiorgio
259 et al., 2002). As discussed above, large gamont stages have been identified by numerous groups,
260 both *in vitro* and *in vivo*. Some studies, which have used both *Cryptosporidium* oocyst wall
261 antibodies such as Cy5-Crypt-a-Glo™ (Waterborne, Inc) and Sporo-Glo™, have reported that
262 novel gamont stages detected in biofims did not label with either type of antibody, suggesting that
263 the surface epitopes that bind Sporo-Glo™ are not expressed by these stages (Koh et al., 2014).
264 Thus it is possible that current detection methods cannot identify all stages of *Cryptosporidium* that
265 may be present in faecal samples and/or in water supplies. However, other cell-free studies have
266 observed labelling of large gamont stages with both Sporo-Glo™ and Cryptocel (Cellabs) and these
267 stages appeared to be surrounded by a thick multi-layered wall which may be able to resist the
268 external environment and disinfection processes (Hijjawi, unpublished observations). Whether
269 current antibodies cross-react with novel gamont-like stages remains to be determined, but as
270 gamont stages are much larger than *Cryptosporidium* oocysts (10-35 µm v 5-8 µm), any observed
271 cross-reaction is likely to be dis-regarded. The fact that novel gamont-like stages have been
272 identified in faecal samples in large numbers (Hijjawi et al., 2002), suggests that this is not an
273 abnormal development as a result of a lack of a host cell, but may be a normal part of the
274 *Cryptosporidium* life cycle.
275
276 *4.2 What is the susceptibility of these novel stages to disinfection?*
277
278 Studies conducted to assess the survival of *Cryptosporidium* under environmental conditions
279 have focussed on oocysts only (Kothavade, 2012). The die-off rate of *Cryptosporidium* oocysts in
280 water is 0.005–0.037 10log-units day^{-1} under natural environmental conditions (Medema et al.,

2006), but the fate of these novel life-cycle stages in water and the effect of disinfection procedures on novel extracellular stages are unknown.

4.3 Ability of Cryptosporidium to survive and reproduce in biofilms

Biofilms (aggregates of micro-organisms) in both drinking water and wastewater distribution networks represent a potentially significant, long-term reservoir of *Cryptosporidium* because biofilm properties potentially allow them to trap and progressively concentrate *Cryptosporidium* oocysts, prior to releasing them in bulk upon dislodgment of biomass (Lazarova and Manem, 1995). Biofilms therefore have the potential to hold large quantities of oocysts that may not be represented in water samples, potentially resulting in contamination of source waters that may have been classified as oocyst free and safe for human exposure (Angles et al., 2007; DiCesare et al., 2012a). Indeed, the release of *Cryptosporidium* oocysts back into the surrounding water has been shown to be responsible for frequent sporadic *Cryptosporidium* outbreaks (Howe et al., 2002; Wingender and Flemming, 2011). Biofilm erosion also explains the appearance of oocysts in water distribution systems long after a contamination event and were suggested to be the reason for ongoing recoveries of oocysts from a drinking water distribution system, following a waterborne cryptosporidiosis outbreak in England (Howe et al., 2002). Biofilms have also been shown to reduce solar disinfection of *Cryptosporidium* (DiCesare et al., 2012b), however, whether biofilms would provide protection to *Cryptosporidium* from predation by natural zooplankton such as rotifers remains to be determined.

As *Cryptosporidium* was believed to be an obligate intracellular parasite, it was thought to lack the capacity to proliferate within biofilms and studies had focused only on the association of the oocyst stage within biofilms (Keevil, 2003; Searcy et al., 2006; Wolyniak et al., 2009; Wendt et al., 2015). Recently however, *Cryptosporidium* developmental stages (sporozoites, trophozoites,

large meronts, merozoites, microgamonts, gamont-like cells and extra-large gamont-like cells) have been identified from artificial biofilms using various techniques including scanning electron microscopy (SEM) and flow cytometry (Koh et al., 2013; 2014). When exposed to an artificial biofilm environment *Cryptosporidium* oocysts initiated excystation and released viable sporozoites, suggesting that biofilm-derived factors are therefore sufficient to initiate the excystation process (Koh et al., 2014). Both asexual and sexual life cycle stages similar to the host-based life cycle were identified but production of new oocysts was not reported (Koh et al., 2014). Extra-large "gigantic" gamont-like cells (30 × 35 μm) were also detected in biofilms and it is thought that they may have a role in producing infective stages of *Cryptosporidium* in biofilms (Koh et al., 2014; Clode et al., 2015). These very large stages have also been reported in cell-free culture and appeared to have a thick outer wall (Hijjawi et al., 2002 and unpublished observations). As discussed above, it is possible that mature oocysts can emerge from these gigantic stages but further research is required.

How *Cryptosporidium* obtains nutrients to grow in biofilms is not known but as discussed above, the ability of *Cryptosporidium* to increase its surface area to optimise surface-mediated nutrition (Aldeyarbi and Karanis, 2016c) may be involved. Surface nutrition through dispersed micropores (pinocytosis) or osmotrophic nutrition via hair-like microvilli has been proposed in gregarine species (Warner, 1968; Prensier et al., 2008), and it is possible that similar mechanisms are also present in *Cryptosporidium*. However, whether biofilms or water will have the necessary precursors that *Cryptosporidium* normally need to scavenge from the host, because they lack the relevant biosynthetic pathways (Abrahamsen et al., 2004), is another important issue that needs to be investigated.

4.4 Implication for modelling the fate and transport of Cryptosporidium

A number of variables can be considered for modelling the fate and transport of *Cryptosporidium* within aquatic domains (Walker and Stedinger, 1999). So far algorithms have been successfully applied to only one *Cryptosporidium* life stage, with pre-determined physical–chemical– and biological–characteristics: the thick-walled oocyst. This has been assumed to have specific coefficient parameters, for instance, for buoyancy, weight, size, settling velocity, sensitivity to UV light and chlorine, biological activity (i.e., ability to replicate) etc. Clearly, the validity of the numerical models and algorithms currently available must be revised on the basis of potentially different properties, peculiar to the novel life stages.

5 Research needs

In addition to the industry research needs outlined below, basic research to better understand the extracellular novel life cycle stages is needed. The novel stages have already been examined by light microscopy (Hijjawi et al., 2002; Hijjawi et al., 2004; Rosales et al., 2005; Koh et al., 2014), immunolabelling, confocal and scanning electron microscopy (SEM) (Edwards et al., 2012; Koh et al., 2014), transmission electron microscopy (TEM) (Rosales et al., 2005; Aldeyarbi and Karanis, 2016a) and laser microdissection coupled with PCR and sequencing (Hijjawi et al., 2002). Novel gamont stages have also been purified *in vivo* from mice infected with *C. parvum* and cattle naturally infected with *C. andersoni* (Hijjawi et al., 2002; Hijjawi et al., 2004). It is still unclear however, where in the life cycle these stages appear, but it has been suggested that they derive from trophozoites, with “a possible role in the generation of more trophozoites or merozoites for new oocyst production in host cell-free and aquatic environments” (Clode et al., 2015). The abundance of novel stages in faecal samples and their infectious potential in vitro and in vivo also needs to be understood as well as whether the novel stages are found in all species or only in some. If it transpires that novel stages are shed in low abundance and are not infectious in vitro or in vivo, then

some of the more sophisticated experiments outlined below may not be required. However as noted previously, novel stages have been reported in large numbers in cattle faeces (Hijjawi et al., 2002).

5.1 Disinfection studies

Data on the survival of life cycle stages other than oocysts under the conditions they are exposed to in the natural environment are required to establish the risk posed by these stages. As a key barrier in drinking water supply, the ability of chlorine and chloramines, as well as other disinfectants used by the water industry, to disinfect the various *Cryptosporidium* life cycle stages including novel stages needs to be determined in order to fully assess water quality risks. It is also important to note that not all countries (including some European Union countries) use chemical disinfection or have a disinfectant residual for drinking water supplies. If *Cryptosporidium* can replicate in environmental biofilms, then systems without a residual would be at greater risk.

Experimental viability studies of *Cryptosporidium* oocysts in water have also found that oocysts were still able to transform into trophozoite forms following UV disinfection treatment, although they were not able to develop any further (Belosevic et al., 2001).

5.2 Improvements to the cell-free culture model

Studies have reported that *Cryptosporidium* can complete its life cycle and produce new oocysts in 5-8 days post-culture inoculation (Hijjawi et al., 2004; Aldeyarbi and Karanis, 2016c). However, currently only a five-fold amplification of the parasite in cell-free culture has been reported (Zhang et al., 2009; Hijjawi et al., 2010). This low level of proliferation is also seen in cell cultures conducted with host cells, where it has been shown that host cell detachment and apoptosis are major contributing factors (Widmer et al., 2000a). As cell-free culture is not limited by host-cell

apoptosis, improvements to cell-free culture may result in higher oocyst outputs. Other factors that affect the development and proliferation of *Cryptosporidium* in host cell culture include the excystation protocol, age and strain of the parasite, stage and size of inoculum and culture conditions such as pH, medium supplements and atmosphere (Hijjawi, 2010; Karanis and Aldeyarbi, 2011; King et al., 2011; 2015). It is likely that many of the same factors will affect the proliferation of *Cryptosporidium* in cell-free media. A recent study by Edwinson et al., (2016), suggested that glycoproteins and free Gal/GalNAc facilitated the switch from invasive *Cryptosporidium* sporozoites to replicative trophozoites and therefore increasing the concentration of Gal/GalNAc in the culture medium may enhance *Cryptosporidium* proliferation in *in vitro* culture.

Recently, a continuous cell culture system, based hollow fiber technology, has been developed that can generate very large quantities of oocysts (1×10^8 oocysts ml^{-1} day^{-1}) (Moranda *et al.* 2016). The hollow fiber cartridge is composed of 200 µm diameter polysulfone hollow fibers with a 20 kDa molecular weight cut-off (FiberCell Systems, Inc., Frederick, MD, USA). Hollow fiber technology provides several unique features: (i) a large surface area for metabolite and gas exchange, which are needed for efficient growth of host cells; (ii) the creation of a biphasic medium providing an oxygen rich nutrient supply to the basal layer of the host cells, while permitting the provision of an anaerobic nutrient rich supply to the apical side mimicking the gut; (iii) the ability to obtain high numbers of in vitro cultured *C. parvum* oocysts for biochemical and molecular studies (Moranda *et al.* 2016). The ability to generate the intestinal redox conditions is a critical factor in the success of the method. The medium is based on a modified MEM medium plus serum and additives that promote parasite growth (lipids, redox buffers and vitamins), based on recent biochemical and genetic studies (Abrahamsen et al., 2004; Zhu, 2008; Zhu et al., 2010). For example, *C. parvum* lacks fatty acid synthase II biosynthetic machinery, suggesting they are dependent upon fatty acid salvage from the host (Zhu et al., 2010). In support of this, the yield of parasites obtained from the continuous culture system by Moranda et al. (2016), was significantly

improved by including a lipid supplement, which included the omega-3 fatty acids, alinolenic acid, eicosapentaenoic acid and docosahexaenoic acid. It would be interesting to trial this technology and modified medium for cell-free culture as it may also dramatically increase the oocysts yield in cell-free culture. The ability to culture *Cryptosporidium* in large quantities will be extremely useful in drug assessment and in research on the evolutional biology and invasion mechanisms of *Cryptosporidium*. It will also be of great benefit to the water industry. For example, to date, oocyst disinfection and viability studies have concentrated on *C. parvum* and little is known about survival and disinfection of *C. hominis* or other species that can infect humans as only one study on UV disinfection has been conducted on *C. hominis* (Johnson et al., 2005). This is because only *C. parvum* can be readily cultured in mice (Meloni and Thompson 1996). A gnotobiotic pig model has been established for *C. hominis* (Widmer et al., 2000b; Pereira et al., 2002) and *C. hominis* is also reportedly capable of infecting immuno-suppressed gerbils (Baishanbo et al., 2005); however, this is beyond the facilities of most laboratories and the use of animals also has ethical implications.

5.3 Development of gamont and stage-specific antibodies

New antibodies that target more of the *Cryptosporidium* developmental stages are needed to further investigate the life cycle of *Cryptosporidium*. Mono– and poly–clonal antibodies against *Cryptosporidium* oocysts are available from a wide range of global suppliers. All these products target one or more antigens expressed on the oocyst wall and are widely used in a variety of ways, including immunofluorescence microscopy, flow cytometry (FCM), laser scanning-based systems, and immunomagnetic separation (IMS methods).

Oocyst wall antigens are composed predominantly of *Cryptosporidium* oocyst wall proteins (COWP1- COWP8), a family of proteins that contain polymorphic Cys-rich and His-rich repeats (Chatterjee et al., 2010). There are also knob-like structures on the inner surface of *C. parvum*

oocyst walls, which cross-react with an anti-oocyst monoclonal antibody (Entrala et al., 2001). To the best of our knowledge, Sporo-Glo (Waterborne Inc., USA) is the only commercially-available product targeting *Cryptosporidium* antigens specific for sporozoites and other intracellular life cycles stages. Cross-reactivity of the currently available commercial products, with antigens or epitopes expressed on the novel life stages, has not been tested and certainly warrants further investigation.

5.3 Evaluation of the ability of Cryptosporidium to survive and propagate in biofilms

Genomic studies have shown that *Cryptosporidium* has lost the de novo biosynthetic capacity for purines, pyrimidines, and amino acids and relies solely on scavenge from the host via a series of transporters (Abrahamsen et al., 2004). Previous studies by Koh et al. (2013; 2014), demonstrated the attachment and development of *Cryptosporidium* in an artificial biofilm (*Pseudomonas aeruginosa)*, suggesting that like gregarines, *Cryptosporidium* is very plastic in its response to its environment and is able to survive and grow on nutrients released by biofilms. Studies are required to investigate under what conditions *Cryptosporidium* can attach to and multiply in bacterial biofilms representative of biofilms found in activated sludge in water and wastewater utilities. This should include identifying the conditions in biofilms that trigger oocyst excystation, determining if bacteria in natural biofilms can provide *Cryptosporidium* with the required purines, pyrimidines, and amino acids for survival and replication and if new thick-walled environmentally robust oocysts are formed in biofilms. Additional studies may show that *Cryptosporidium* development in biofilms is minimal and therefore not a significant risk, however it is important that this research is conducted to better inform risk models.

6 Conclusions

455

456 There is now growing evidence that the gregarine *Cryptosporidium* can excyst and multiply
457 without undergoing host cell encapsulation and epicellular development, and research in this area is
458 essential for more effective catchment management. The prevalence and environmental robustness
459 of gamont stages in the environment and ability of currently used antibodies to detect gamont stages
460 needs to be explored. Similarly, whether oocysts can be produced in biofilms and their infectivity
461 also needs to be examined.

462

463 **Acknowledgements**

464 The authors are grateful for funding for our current *Cryptosporidium* research from the Australian
465 Research Council Linkage Grant number LP130100035.

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808 Fig. 1. (a) Nomarski interference-contrast photomicrograph of an extracellular gamont-like stage
809 purified from mice after 72 hrs infection with *C. parvum* and (b) two gamont-like stages fused
810 together with two big nuclei confirming their fusion or syzgy, (c) resulting in the formation of a
811 gametocyst (multi-nucleated mass)-like stage, which originated after their fusion. Scale bar = 5µm.
812 Images reproduced with permission from Hijjawi et al., (2004).

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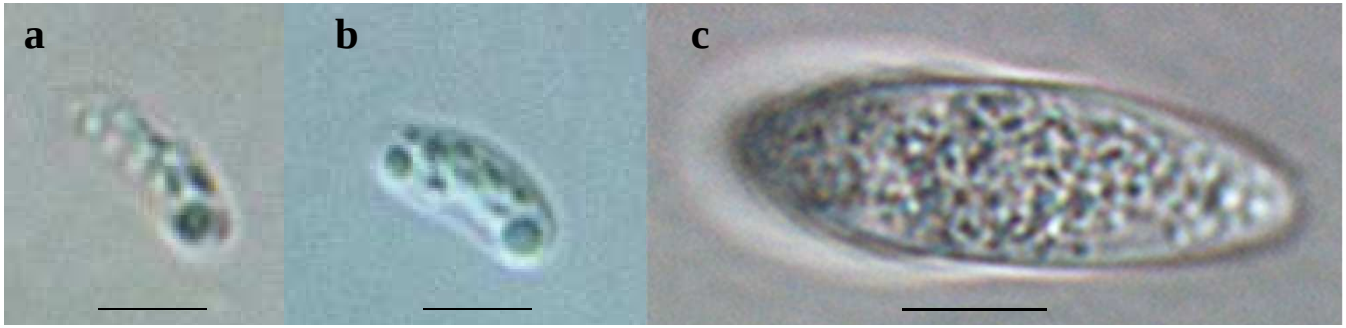
Table 1. Similarities between *Cryptosporidium* and gregarine species.

Properties	*Cryptosporidium*	Gregarines	References
Life cycle	Monoxenous	Monoxenous	Levine, 1977; Fayer and Ungar, 1986; Rueckert et al., 2013
Location within the host cell	Occurs in the intestines enterocyte brush border	Occurs in the intestines enterocyte brush border	Levine, 1984; Fayer, 2008;
Epicellular location	Yes	In some species (Gregarinoidea)	Valigurová et al., 2007; Fayer, 2008
Feeder organelle	epimerite	mucron or epimerite	Huang et al., 2004; Valigurová et al., 2007; Borowski et al., 2008; Wiser, 2011; Aldeyarbi and Karanis, 2016a;
Myzocytosis-like feeding (cytoplasm of the host is taken up by the attached parasite)	Yes	Yes	Valigurová et al., 2007; Karanis and Aldeyarbi 2011; Aldeyarbi and Karanis, 2016a
Extracellular development	Yes	Yes	Hijjawi et al., 2002; Rosales et al., 2005; Karanis et al., 2008; Borowski et al., 2010; Koh et al., 2013, 2014; Huang et al., 2014;
Ability for intracellular or extracellular asexual replication (merogony) of trophozoites	Yes	Yes	Lange and Lord, 2012; Aldeyarbi and Karanis, 2016a; Aldeyarbi and Karanis, 2016b
Undulating epicytic-like folds covering the surface of the extracellular stages	Yes	Described for some species belonging to Terragregarina.	Lucarotti 2000; Butaeva et al. 2006; Valigurová et al., 2007; Rueckert et al. 2011; Desportes and Schrével 2013; Aldeyarbi and Karanis, 2016a
Presence of parasitiphorous sac/vacuole	Double-membrane	Multi-membranous for *Ditrypanocystis* species	Butaeva et al. 2006;Valigurová et al., 2015
Presence of Apicoplast	Absent	Mostly absent	Zhu et al., 2000; Abrahamsen et al., 2004; Cavalier-Smith, 2014
Syzygy (end to end pairing for reproduction)	Present	Present	Beams et al., 1959; Vavra and McLaughlin, 1970; Hijjawi et al., 2002; Kuvardina and Simdyanov, 2002; Toso and Omoto 2007; Borowski et al., 2010; Rueckert et al., 2011; Desportes and Schrével 2013; Aldeyarbi and Karanis, 2016a; Aldeyarbi and Karanis, 2016c
Ability of sporozoites /zoites to develop directly into sexual stages without merogony	Reported for *C. parvum*	Urosporoidea (formerly eugregarines)	Rueckert et al., 2013; Aldeyarbi and Karanis, 2016c
Auto-infective oocysts	Yes	Reported in *Triboliocystis garnhami* and *Farinocystis tribolii*	Dissanaike 1955, Žižka 1972; Fayer, 2008
No. of sporozoites / oocyst	Four	Four in many species	Kuriyama et al., 2005; Fayer, 2008, Wiser, 2011
Presence of the apical complex organelles as conoid and polar rings in	Yes	Reported in *Mattesia grandis*	Vavra and McLaughlin, 1970; Aldeyarbi and Karanis, 2016b

sporozoites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Highlights

- *Cryptosporidium* now grouped with gregarine parasites
- Both epicellular and extracellular development
- Can diagnostic antibodies cross-react with novel stages?
- Susceptibility of novel stages to disinfection?
- Growth in biofilms?