The Interaction Of Cryptosporidium With Aquatic Biofilm Systems

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This thesis is presented for the degree of Doctor of Philosophy at Murdoch University, 2013
Declaration

I declare that this thesis is a true account of my own research and contains work, which has not been submitted for a degree at any other educational institution.

____________________
Wan Hon Koh
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Abstract

Cryptosporidium parvum is a common, opportunistic, diarrhoeal-causing, apicomplexan pathogen in humans, of which water is an important transmission vehicle. Recently, aquatic biofilms have been recognised as environmental reservoirs for the infective stage (oocysts) of Cryptosporidium, yet their fate after being trapped within biofilms is unknown. Previous cell-associated and cell-free studies have demonstrated, controversially, that Cryptosporidium may be able to multiply extracellularly, indicating that Cryptosporidium is not an obligate intracellular microorganism, and that the environment may play an important role in shaping its life cycle. Previously published data raise the question as to whether Cryptosporidium can survive and multiply within biofilms, resulting in an increase in numbers before release into water systems, leading to possible disease outbreaks.

This study, therefore, aimed to investigate the ability of biofilms to support Cryptosporidium multiplication. This was achieved using a combination of quantitative polymerase chain reaction (qPCR), flow cytometry (immunolabeled with Cryptosporidium oocysts-specific antibody), confocal microscopy (immunolabeled with Cryptosporidium developmental stage-specific antibody) and scanning electron microscopy (SEM) techniques. To mimic a water distribution system, Pseudomonas aeruginosa biofilm flow cell systems were established and unexcysted C. parvum oocysts were constantly supplied over a 6-day period. Prior to analysis, the four analytical methods were designed and empirically optimised according to the nature of the experimental sample studied.

Quantitative PCR results showed a significant increase (P<0.001) in Cryptosporidium as the biofilm matured, with the total number of C. parvum
multiplying 2-3 fold during this period. Flow cytometry analysis also revealed that the captured oocysts had undergone excystation in biofilms, confirming that the increase in *Cryptosporidium* number was due to *Cryptosporidium* multiplication. From this, various *Cryptosporidium* developmental stages (sporozoites, trophozoites, meronts, and merozoites) were also identified from the biofilm using confocal microscopy and SEM. A correlative study using both SEM and confocal imaging determined that the observed developmental stages were *Cryptosporidium*, rather than degenerate/accumulated oocysts or yeast contamination. Furthermore, SEM analysis also revealed that *Cryptosporidium* may form a parasitophorous vacuole independently, potentially allowing it to complete its life cycle extracellularly. In addition, certain stages of the *Cryptosporidium* life cycle (trophozoites, meronts, and some previously undescribed gamonts) in biofilms were identified, and shown to closely resemble stages reported in the gregarine life cycle, emphasising the possibility that *Cryptosporidium* has inherited the capability to multiply extracellularly from their gregarine ancestor.

In conclusion, this study has successfully shown that biofilms can support *Cryptosporidium* multiplication in aquatic environments and thus, also demonstrated a role for biofilms in outbreaks and spreading of this disease. The generated results are novel, offering new insights into the role of biofilms in the *C. parvum* life cycle, providing additional information for water authorities, aiding in the control of *Cryptosporidium* and biofilm-contaminated drinking water.
Publications

Part of the work presented in this thesis has been published and accepted for presentation in scientific conferences as described below:


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<th>Description</th>
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<tbody>
<tr>
<td>%</td>
<td>percentage</td>
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<tr>
<td>μg</td>
<td>Microgram(s)</td>
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<tr>
<td>μL</td>
<td>Microlitre</td>
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<td>μm</td>
<td>Micrometer</td>
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<td>μM</td>
<td>Micromolar</td>
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<td>B</td>
<td>Biofilm(s)</td>
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<tr>
<td>BF</td>
<td>Biofilm-free</td>
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<tr>
<td>BO</td>
<td>Biofilm-only</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BSD</td>
<td>Backscattered electron detector/detection</td>
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<tr>
<td>C. parvum</td>
<td>Cryptosporidium parvum</td>
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<tr>
<td>CF</td>
<td>Cell-free</td>
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<tr>
<td>cm</td>
<td>Centimetre(s)</td>
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<tr>
<td>CT</td>
<td>Cycle threshold</td>
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<tr>
<td>DNA</td>
<td>Desoxyribonucleic acid</td>
</tr>
<tr>
<td>et al.</td>
<td>And others</td>
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<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
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<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>g</td>
<td>Gravitational force</td>
</tr>
<tr>
<td>h</td>
<td>Hour(s)</td>
</tr>
<tr>
<td>HCT-8</td>
<td>Human ileocecal tumor (adenocarcinoma) epithelial cell line</td>
</tr>
<tr>
<td>IS</td>
<td>Interkingdom signalling</td>
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<tr>
<td>mg</td>
<td>Milligram(s)</td>
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<tr>
<td>mM</td>
<td>Milimolar</td>
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<tr>
<td>nM</td>
<td>Nanomolar</td>
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<tr>
<td>NTC</td>
<td>Negative control</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>pH</td>
<td>Power of hydrogen</td>
</tr>
<tr>
<td>PS</td>
<td>Parasitophorous sac</td>
</tr>
<tr>
<td>PV</td>
<td>Parasitophorous vacuole</td>
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<tr>
<td>Quantum Dot(s)</td>
<td>QD(s)</td>
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<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
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<tr>
<td>QS</td>
<td>Quorum sensing</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------------------------------------------</td>
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<tr>
<td>R²</td>
<td>Coefficient efficiency</td>
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<tr>
<td>RPMI</td>
<td>Rapid prototyping and manufacturing institute</td>
</tr>
<tr>
<td>S</td>
<td>Stock</td>
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<tr>
<td>SE</td>
<td>Secondary electron</td>
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<td>Sec</td>
<td>Second(s)</td>
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<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>TSB</td>
<td>Tryptic soy broth</td>
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<td>V</td>
<td>Volts</td>
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<td>W</td>
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1

General Introduction
1.1 Summary

Due to its resistance to chlorine, Cryptosporidium is one of most difficult to control waterborne microorganisms. Therefore, robust catchment and treatment barrier management practices (water filtration) have been implemented over the years to minimise the potential of oocysts entering treated drinking water systems. However, when this system is poorly managed, absent or breached, oocysts will enter into the drinking water distribution system and encounter biofilms. Nevertheless, the potential risk posed by Cryptosporidium in association with biofilms remains unknown.

Biofilms are not only found in water distribution systems but also in various aquatic environments such as ponds and rivers. These aquatic biofilms have been shown to serve as environmental reservoirs for Cryptosporidium oocysts (Searcy et al., 2006; Wolyniak et al., 2009, 2010). However, investigations into Cryptosporidium ecology have mainly been focused upon the physical factors that affect Cryptosporidium retention within biofilms (Wolyniak et al., 2009, 2010). The ability of biofilms to support the growth and development of Cryptosporidium has yet to be determined. The oversight in this field of research may be due to the conventional belief that Cryptosporidium can only multiply intracellularly, despite increasing evidence to suggest that their extracellular capability cannot be disregarded (Hijjawi et al., 2004; Boxell et al., 2008; Zhang et al., 2009; Hijjawi et al., 2010). This review will, therefore, focus on the extracellular development of Cryptosporidium and the importance of understanding the role biofilms may play in the life cycle of Cryptosporidium and in showing the relationships between gregarines and Cryptosporidium.
1.2 *Cryptosporidium*

*Cryptosporidium* is one of the most common enteric protozoan parasites that infect both human and animals (Thompson et al., 2005). Animal to human transmission of *Cryptosporidium* (*i.e.* *C. parvum*) is a public health concern (De Graaf et al., 1999; Thompson et al., 2008). *Cryptosporidium* was first described by Ernest Edward Tyzzer in 1907 (Fayer, 2008) but was only recognised as a pathogen after an outbreak of diarrhoea in a turkey flock in 1955 (Carey et al., 2004). However, it was not recognised to be of medical significance until the first human case was reported in 1976 in an immuno-suppressed (HIV) patient (Meisel et al., 1976). The public health impact of *Cryptosporidium* became prominent when a massive outbreak of acute watery diarrhoea occurred in Milwaukee, USA in 1993 (Mac Kenzie et al., 1994; Corso et al., 2003). Since then, *Cryptosporidium* has rapidly become recognised as one of the most serious and difficult to control waterborne pathogens to date (Ramirez et al., 2004). Furthermore, the direct medical costs and productivity losses for *Cryptosporidium* infections associated with the Milwaukee outbreak was $96.2 million: $31.7 million in medical costs and $64.6 million in productivity losses. The average total costs for persons with mild, moderate, and severe illness were $116, $475, and $7808, respectively (Corso et al., 2003). The high economical costs resulting from a single *Cryptosporidium* outbreak has brought about a boom in *Cryptosporidium* basic biology research with emphasis on developing methods for recovery and detection of the oocyst stage, and prevention and treatment of the disease (Fayer, 2008). However, as these interventions and control strategies aren’t clear and therefore the need for having a detailed understanding of the *Cryptosporidium* life cycle and behaviour isn’t clear.
1.2.1 Life Cycle

The generally accepted life cycle of Cryptosporidium is similar to other coccidian parasites, which involves the processes of merogony (asexual phase), gametogony (sexual phase), and sporogony within a host, and the release of thick-walled oocysts into the environment (Figure 1.1) (Thompson et al., 2005). The life cycle of Cryptosporidium begins with the ingestion of viable and infective oocysts by a susceptible host (Collinet-Adler and Ward, 2010). The changes in temperature, pH, presence of bile salts and pancreatic enzymes during the passage through the host’s gut triggers Cryptosporidium excystation (Borowski et al., 2008). This process breaks the suture in the oocyst wall, and four infective sporozoites are released. Sporozoites exhibit apical organelle discharges to degrade intestinal mucus (Borowski et al., 2008) and travel to the infection site by gliding motility, where host-cell invasion is initiated (Current and Reese, 1986). Once the sporozoite adheres to the intestinal cell, host-cell contact via specific receptor-ligands is established and parasitophorous vacuole (PV) formation around the parasite is induced by the extension of the host cell microvillus membrane (Figure 1.2) (Current and Reese, 1986). An electron dense band within the PV separates Cryptosporidium from the host cell cytoplasm, and a feeder organelle is used for nutrient and energy uptake from the host, providing favourable growth conditions for Cryptosporidium (Lumb et al., 1988; Yoshikawa and Iseki, 1992; Beyer et al., 2002; Thompson et al., 2005; Fayer, 2008). Unlike other protozoan such as Trypanosoma, Toxoplasma, Sarcocystis and Leishmania, which form PVs while penetrating deep within the host cytoplasm, PV development by Cryptosporidium on the surface of epithelial cells is
unique and distinguishes the genus *Cryptosporidium* from all other apicomplexans (Beyer *et al.*, 2002).

Figure 1.1: The life cycle of *Cryptosporidium* [adapted from Thompson *et al.* (2005)]
After internalisation, the sporozoite within the PV rounds up and becomes a trophozoite (Current and Reese, 1986). At the late trophozoite development stage, the merogony process leads to the formation of type I meront (Fayer, 2008). During type I meront development, 6 or 8 merozoites form and attach onto a small residual body at the periphery of the PV (Lefkowitch et al., 1984). The splitting of the PV membrane causes the detachment of merozoites, allowing their escape into the gut lumen to infect other intestinal cells and to develop into new trophozoites/type I meronts or further develop into type II meronts (Bird and Smith, 1980). Type II meronts usually contain 4 merozoites that are frequently arranged in parallel on a residuum at the base of the PV. The rupturing of the PV releases type II merozoites that are responsible for the gametogony/sexual reproduction stages (Current and Long, 1983; Fayer, 2008). After adhering to the epithelial surface, type II merozoites trigger PV formation and further differentiate into either microgamonts (male) or macrogamonts (female) (Vetterling et al., 1971). Many microgametes are produced within a single microgamont. The free microgametes display jerky forward gliding.
movements and are often attached to macrogamont for fertilisation (Current and Reese, 1986). The fertilised macrogamont then undergoes the sporogony process to reproduce 4 sporozoites. Once a mature oocyst is formed in the PV, it is released into external environment.

Two different types of oocysts are produced in the Cryptosporidium life cycle: thick- and thin-walled oocysts. Thick-walled oocysts are responsible for the external transmission stage of the life cycle (Thompson et al., 2005). It is extremely resistant to chemical treatment and can survive for several months in the external environment (Davies et al., 2004; Thompson et al., 2005). On the other hand, thin-walled oocysts are responsible for the auto-infective stages within the infected host.

1.2.2 Intracellular, Epicellular and Extracellular

With the tremendous improvement in in-vitro, cell-free cultures and in-vivo techniques, Cryptosporidium life stages have been revealed to be more complicated than initially believed. Prior to the discovery of its extracellular capability, Cryptosporidium had always been assumed to be an intracellular parasite, even though it neither penetrates under the host cell membrane nor does it have close contact with the host cell cytoplasm (Figure 1.3) (Petry, 2004; Petry et al., 2009). The term “intracellular but extracytoplasmic” is used in most studies due to the belief that the PV was formed by the extension of the host microvillus and that Cryptosporidium was connected to the host cell by a tunnel (O'donoghue, 1985; Smith and Rose, 1998; Tzipori and Griffiths, 1998; Abrahamsen et al., 2004; Huang
et al., 2004). However, this tunnel was shown to disappear once a feeder organelle and electron dense band formed (Valigurová et al., 2008).

The concept of Cryptosporidium being an “epicellular” parasite has been also introduced and more detailed structures revealed (Valigurová et al., 2008) (Figure 1.3). At the same time, it is also proposed that the term PV, for its location in Cryptosporidium spp., is misleading because this term refers solely to the vacuolar space bordered by a membrane (Valigurová et al., 2008). The term parasitophorous sac (PS) was proposed by Valigurová et al. (2008) to be more suitable for describing Cryptosporidium because the PS was revealed to be more than just a feeder organelle and dense band. Valigurová et al. (2008) stated that the PS is composed of (i) host-parasite space; (ii) a host cell membrane fold continuous with the microvillus membrane of the gastric cell, which also indicates that the microvillus does not have a role in PS formation; and (iii) a thin layer of host cell cytoplasm enclosed by this membrane fold. These new observations further show that Cryptosporidium only remains attached to the host cell surface and no other intimate association between Cryptosporidium and the host cytoplasm has been found.

More recently, there has been increasing evidence to suggest that in the absence of a PV, Cryptosporidium can also multiply extracellularly on the surface of the epithelial cells in in-vitro culture and in-vivo (Hijjawi et al., 2002; Borowski et al., 2010) (Figure 1.3). These observations have been further supported by the identification of extracellular developmental stages of Cryptosporidium in cell-free cultures (Hijjawi et al., 2004; Karanis et al., 2008; Zhang et al., 2009; Hijjawi et al., 2010). However, it appears that the life cycle of Cryptosporidium in cell-free culture is dissimilar to the life cycle observed from in-vitro culture and in-vivo. These include the presence
of large numbers of aggregated merozoites in grape or rosette shapes representing type I and II meronts (Hijjawi et al., 2004; Hijjawi et al., 2010).

Two new unrecognised extracellular stages of *C. parvum* were also noted in cell-free cultures (Hijjawi et al., 2002). These two extracellular stages exhibit different morphologies and are considered relatively big when compared to other known stages, and hence are temporarily referred to as 1 and 2 gamont-like stages. Stage 1 gamont-like cells are spindle shaped (~5 µm) and contain numerous granules, while stage 2 cells are blunt ended rods (~10 µm) (Hijjawi et al., 2002; Hijjawi et al., 2004). The role of these two gamont-like cells in the *Cryptosporidium* life cycle remains unknown but their presence demonstrates that *Cryptosporidium* is not an obligate intracellular parasite (Hijjawi et al., 2002). More importantly, the *Cryptosporidium* life cycle in cell-free culture has unusual developmental plasticity, with the ability to skip the merogony stage and initiate mitotic division from fused sporozoites (Barta and Thompson, 2006). The observation of the *Cryptosporidium* life cycle in cell-free culture not only highlights that induction of the PV formation is solely for protection and competitive purposes in the host but also demonstrates that they are a highly adaptable pathogen that can alter their life cycle according to their surrounding environment for better survival in a way similar to their gregarine ancestors (Valigurová, 2012).
1.3 Gregarines

1.3.1 Life Cycle

Gregarines are single celled parasites that inhabit the intestine and other extracellular spaces of invertebrates (Leander, 2008). They are generally basal to other apicomplexan parasites and hence are regarded as “primitive” cells (Carreno et al., 1999; Leander, 2008; Clopton, 2009). Although they do not have a recognised impact on human welfare, their close relationship with Cryptosporidium may help to improve the understanding of the fundamental properties of Cryptosporidium (Carreno et al., 1999). Gregarines have monoxenous life cycles in invertebrate hosts and exhibit remarkably diverse morphology and behaviour (Leander et al., 2003b).

The life cycle of gregarines (Figure 1.4) begins with the oral ingestion of oocysts from the external environment (Leander, 2006). Sporozoites are then released from
the oocyst and adhere to the surface of the host tissue (epithelium of the intestinal tract). The sporozoite then develops a fixative apparatus called a mucron or epimerite, which further develops into a large trophozoite (Leander et al., 2003b). Mucrons often formed by aseptate gregarines that have less distinctive structures as compared to epimerites that are formed by septate gregarines (Leander et al., 2003b). Septate gregarines are usually refer to gregarines with a transverse superficial septum that demarcates the trophozoite cell into two pseudo-compartments (Rueckert et al., 2010). The role of the mucron or epimerite is similar to that of the Cryptosporidium feeding organelle (Barta and Thompson, 2006). Mature trophozoites will then detach from their host cell anchor and establish contact with other unattached mature trophozoites in a union known as syzygy, which marks the onset of sexual reproduction (Leander et al., 2003b). The syzygy process is believed to increase the genetic diversity of gregarines prior to the sexual reproduction stage (Landers, 2001; Toso and Omoto, 2007). A gametocyte wall forms around each pair of trophozoites, which then begins to divide into hundreds of gametes. Gametes from different gamonts fuse to form zygotes for oocyst production, which are then released via the host’s faeces, or through death or decay (Leander, 2006; Leander, 2008).
1) Released sporozoites adhere to the gut epithelium. 2) Formation of epimerite/mucron during trophozoite transformation process. 3) Mature trophozoites detach from the cell and pair up with other unattached trophozoites by syzygy. 4) Gametocyte wall forms around the paired trophozoites. 5) Trophozoites within gametocyte begin the multiplication process to form gametes. 6) Gametes from different gamonts fuse to form zygotes for oocysts production. 7) Release of oocysts into the environment through host faeces or decay. 8) Ingestion of oocysts by susceptible host.

1.3.2 Gregarine Species Identification

Based on host range, habitat and trophozoite morphology, gregarines are classified into 3 orders, i) archigregarines, ii) eugregarines and iii) neogregarines (Leander, 2008). Archigregarines are found exclusively in marine habitats, are aseptate, and possess intestinal trophozoites that are similar in morphology to infective sporozoites (Leander et al., 2003b; Leander, 2008). They are inferred to be the representative of the most ancestral form of gregarine (Cox, 1994). Eugregarines are believed to be evolved from archigregarines and are found in marine, freshwater and terrestrial habitats (Cox, 1994). They generally lack merogony asexual division in their life cycle and possess large trophozoites that are significantly different in morphology and behaviour to that of sporozoites (Leander et al., 2003b; Leander, 2008).
Neogregarines are also evolved from archigregarines but are the most derived gregarine. They have reduced trophozoites and are found mainly in terrestrial hosts such as insects (Cox, 1994; Leander et al., 2003a; Leander, 2008).

To date, it has been confirmed that species delineation based on these three factors - host, habitat and trophozoite morphology - alone are insufficient and problematic (Rueckert et al., 2011). This is because it was found that the size, shape, and ultrastructure of trophozoites of gregarines are often highly variable depending on different developmental stages of parasite and host environmental conditions (e.g., starved host vs. a fully nourished host) (Rueckert et al., 2011). For example, a wide variety of trophozoite morphotypes have been documented for eugregarines such as *Lecudia tuzetae*, which reflect different growth conditions they are exposed to (Rueckert et al., 2011). This is similar to that observed in the *Cryptosporidium* life cycle, which also has variable morphotypes in different environments (Current and Long, 1983; Current and Reese, 1986; Current et al., 1986; Hijjawi et al., 2001; Hijjawi et al., 2002; Hijjawi et al., 2004; Valigurová et al., 2008; Borowski et al., 2010; Hijjawi et al., 2010).

### 1.3.3 *Cryptosporidium* and Gregarines

Phylogenetic studies show that *Cryptosporidium* forms a monophyletic clade with neither internal coccidian cyst-forming eucoccidia nor with an intermediate sister group to the coccidian. In fact, it has the closest phylogenetic affinity with gregarines (Carreno et al., 1999; Zhu et al., 2000; Morrison et al., 2004). *Cryptosporidium* spp. is most closely related to the archigregarine, *Selenidium* spp., and is, therefore, representative of one of the earliest diverging apicomplexans (Carreno et al., 1999;
Leander et al., 2003a). This, together with its close phylogenetic affinity to gregarines, may explain where Cryptosporidium attains the capability to multiply extracellularly, and also exhibit variable morphological developmental stages in different environments (in-vitro, cell-free cultures and in-vivo).

Furthermore, the Cryptosporidium PV is similar to the gregarine epimerite or mucron in that it is confined to the apical surfaces of intestinal epithelia cells (Barta and Thompson, 2006). However, in contrast to the epimerite/mucron, an electron dense band and feeder organelle is produced by Cryptosporidium to separate itself from the host cytoplasm and to regulate nutrient uptake from the host cell. It has been suggested that this feeding mode is an evolutionary modification from the ancestral myzocytotic morphological adaptation (Barta and Thompson, 2006). Myzocytosis is used by dinoflagellates and primitive apicomplexan forms such as Selenidium spp. for micropredation and parasitic infection (Figure 1.5) (Barta and Thompson, 2006). This method is known as “cellular vampirism” in which the predatory cell pierces the cell wall/membrane of the host cell with a feeding tube and sucks out the cellular contents (Leander, 2008). During evolution, the replacement of the feeding tube with the feeding organelle and dense band further shows that Cryptosporidium is not intimately dependent on the host cell (Figure 1.5). Perhaps the sole function of PV formation by Cryptosporidium is to allow it to adsorb nutrients more efficiently from the host and to protect itself from the host immune system.

A previous study has highlighted that the difference in the development cycles between gregarines and Cryptosporidium is due to the lack of a syzygy stage in Cryptosporidium (Carreno et al., 1999). However, this difference has been questioned by recent Cryptosporidium in-vitro and cell-free culture studies (Hijjawi
Pairing of trophozoite stages has frequently been observed. Furthermore, the life cycle of *Cryptosporidium* in cell-free culture has been shown to be remarkably similar to the two *Mattesia* species of gregarine, *M. dispora* and *M. germinate* (Levine, 1985; Kleespies *et al.*, 1997). Their life cycles were compared in a detailed review by Thompson *et al.* (2005). The previously unrecognised gamont-like stages in cell-free culture were also shown to be similar to the trophozoite/gamont stages described in gregarines (Levine, 1984; Petry, 2004). The revelation of striking similarities between the *Cryptosporidium* life cycle to that of gregarines and the ability to reproduce extracellularly may provide new insights in environmental ecology for the control of *Cryptosporidium* in water.

![Figure 1.5: Comparison of myzocytosis and PV formation [adapted from Barta and Thompson (2006)].](image)
1.4 Water Supply and *Cryptosporidium*

*Cryptosporidium* is mainly transmitted by water, typically via recreational or drinking water (Fayer, 2008). To date, most *Cryptosporidium* outbreaks associated with drinking water are caused by source water contamination with animal sewage (Fayer, 2004). Agricultural animal waste has become a threat to the water industry as farms with domestic animals often excrete large numbers of oocysts in their faeces (Rose, 1997; Fayer, 2004). Although agricultural waste appears to be an important cause of *Cryptosporidium* waterborne outbreaks, *Cryptosporidium* outbreaks can also be due to contaminated human waste, as demonstrated by the outbreak in Milwaukee, USA (Rose, 1997). Infected humans and animals usually can pass up to 10 billion sporulated and infectious *Cryptosporidium* oocysts per gram of faeces (Smith and Rose, 1998; Fayer, 2004). The runoff after rainfall events carry these oocysts into surface waters, leading to the occurrence of sporadic and massive outbreaks if water treatment is not effective (Rose, 1997).

Piped water is generally obtained from surface and ground waters (Percival *et al.*, 2000). Surface waters include lakes, ponds, rivers and streams while groundwater is water present in fully saturated soils and rocks (Percival *et al.*, 2000). When compared to ground water, surface water is easily located and extracted, but it is also readily affected by surrounding environmental factors, such as agrochemical use and faecal contamination (Percival *et al.*, 2000). Hence, *Cryptosporidium* is more often detected in surface waters than in groundwater (Ritter *et al.*, 2002). Conventional surface water treatment procedures that include coagulation, flocculation, sedimentation, filtration and chemical treatment have been successful in removing or inactivating *Cryptosporidium* present in water distribution systems (Fairley *et al.*, 2000).
1999). However, under conditions when these barriers are less effective, fail, or are not present at all, *Cryptosporidium* may present a risk to the water distribution system, especially when they encounter environmental aquatic biofilms in the natural environment.

### 1.5 Aquatic Biofilms

Biofilms are assemblages of microorganisms encased by a biofilm matrix that function as a cooperative consortium (Costerton *et al*., 1999; Davey and O'toole, 2000). Bacteria can obtain many benefits from forming biofilms, including (i) protection of residing microorganisms from harmful conditions, (ii) establishment of a nutrient-rich area, and (iii) antimicrobial resistance (Percival and Walker, 1999; Jefferson, 2004). Biofilms can be found everywhere that is in contact with non-sterile water ranging from the most sybaritic (the human mouth), to the most severe (the dry valleys of the Antarctica), which is cogent evidence of their success (Snelling *et al*., 2006; Wingender and Flemming, 2011). Biofilms represent biological systems with a high level of organisation where bacteria can interact with one another chemically and physically to form structured, coordinated and functional communities (Decho, 2000; O'toole *et al*., 2000; Stoodley *et al*., 2002b).

#### 1.5.1 Life Cycle

Biofilms are comprised of either single or multiple microbial species of bacteria and form on a range of biotic and abiotic surfaces (Davey and O'toole, 2000). Mixed-
species biofilms often form in most environments in an organised array, while single species biofilms exist in a variety of infections and on the surface of medical implants (O’toole et al., 2000). Biofilms are heterogeneous and are comprised of aggregates of microbial cells within a biofilm matrix, interstitial voids and water channels (Xi et al., 2006) (Figure 1.6). Water channels are used to separate the microcolonies to circulate nutrients and exchange metabolic products with bulk fluids (Stoodley and Lewandowski, 1994). Furthermore, water channels and voids can also act as gene pools allowing for genetic acquisition and exchange by horizontal transfer (Clutterbuck et al., 2007).

Biofilms are structurally complex, organised dynamic systems and their growth can be depicted as a series of discrete stages in a life cycle (Figure 1.7) (Nadell et al., 2008). Growth begins when planktonic cells make contact with a surface either by Brownian motion, sedimentation or by taxis towards a chemical gradient (Palmer et al., 2007; Nadell et al., 2008). Initial attachment is often reversible so that cells can depart from a surface if conditions change (Nadell et al., 2008). After reversible attachment, irreversible attachment occurs through the use of bacterial flagella activity and chemotaxis. Extracellular pili enable bacteria to position themselves in an act similar to that of grappling hooks, allowing bacteria to grow and divide to form dense cell groups (Figure 1.8) (Palmer et al., 2007; Nadell et al., 2008). At the same time, bacteria will also generate adhesins to enhance their attachment on the surface and also to promote the adhesion of other planktonic microorganisms, forming aggregates on the substratum (Rickard et al., 2003). Most importantly, these aggregates are further strengthened by the production of a biofilm matrix which engulfs them and initiates the biofilm maturation process (Figure 1.9) (Snyder et al.,
2009). As biofilms mature, the overall density and complexity of biofilms increase as surface-bound microorganisms begin to replicate actively (Dunne, 2002).

Biofilms then detach when the availability of nutrients become limited, a critical mass is reached, flow shear or induced by cell to cell signalling process (Dalton et al., 1996; Stoodley et al., 2002a; Hall-Stoodley and Stoodley, 2005). Two processes are known to be involved in cell detachment, erosion and sloughing (Stoodley et al., 2001; Telgmann et al., 2004). Erosion refers to the continual detachment of small portions of biofilm whereas sloughing is a rapid, massive loss of cells from the biofilm together with the biofilm matrix (Stoodley et al., 2001). These two processes allow microorganisms to escape from the biofilms to colonise other surfaces with continuing cycles of adhesion, growth, maturation, and detachment.

Figure 1.6: The heterogeneous population of biofilms (Reprint permission obtained from Centre for Biofilm Engineering).
Figure 1.7: The life cycle of biofilm (Reprint permission obtained from Centre for Biofilm Engineering).

Figure 1.8: The usage of flagella (arrowhead) and pilli (arrow) for the initial attachment of *Pseudomonas aeruginosa* bacteria on the surface. Scale bar: 3 µm. Image taken by Wan Koh, Murdoch University.
1.5.2 The Role of the Biofilm Matrix

The biofilm matrix is essential for the development of a biofilm’s architecture by providing sufficient mechanical stability to maintain a spatial arrangement of the microconsortia over a prolonged period (Flemming et al., 2007; Flemming, 2009). The matrix comprises polysaccharide, proteins, glycoprotein, glycolipid and extracellular DNA (Flemming et al., 2007). All biofilms matrices are highly hydrated, keeping the microorganisms together and retaining water (Wotton, 2005; Flemming et al., 2007; Flemming and Wingender, 2010). The matrices also play a role in interacting with the environment, such as acting as a diffusion barrier to nutrients and cellular products (Allison, 1998). At the same time, they act as a physical barrier to prevent the access of antimicrobial agents and the penetration of
UV light (Del Pozo and Patel, 2007; Wolyniak et al., 2012). It has been shown that biofilms can tolerate antimicrobial agents at concentrations of 10-1000 times higher than needed to kill genetically equivalent planktonic bacteria (Lewis, 2001). Furthermore, the biofilm matrix promotes adherence of other microorganisms onto the biofilm surface, and generates a buffering zone for bacteria to maintain a controlled and favourable microenvironment (Caldwell et al., 1997; Dunne, 2002; Braissant et al., 2007). As such, the biofilm matrix appears to be a highly sophisticated system, which can maintain the multicellular environment and function of the biofilm (Flemming et al., 2007).

1.5.3 Multicellular Behaviour - Creating a Nutrient Rich Microenvironment

By activating intercellular signalling (quorum sensing), biofilms can maximise their productivity, allowing bacteria to communicate with one another and acting as multicellular units in a way similar to cell-cell communication and intercellular signal transduction in eukaryotic systems (Figure 1.10) (Federle and Bassler, 2003; Bassler and Losick, 2006; Hooshangi and Bentley, 2008). Bacteria can adjust their behaviour to their immediate surroundings and compete for limited resources using this quorum sensing system (Jefferson, 2004). Regardless of whether the biofilm is formed from a mixed- or mono-bacterial species, they exhibit substantial heterogeneity, containing segregated subpopulations with different phenotypic and physiological properties (Gilbert et al., 2002; López et al., 2010). Bacteria in biofilms are exposed to an array of distinct environmental signals and are capable of using multiple growth substrates to release numerous extracellular products to
enhance nutrient availability (Davey and O'toole, 2000). In the deepest region of mature biofilms that cannot access water channels, diffusion is the mechanism by which solute transport occurs (Stewart, 2003; Charbonneau et al., 2006), thereby generating a complex chemical gradient with numerous microniches within the same biofilm (López et al., 2010). This heterogeneous behaviour is due to bacteria altering their behaviour according to local conditions (Davey and O'toole, 2000). Usually, cells near the biofilm surface utilise aerobic metabolism while cells within the biofilm must switch to anaerobic metabolism (Figure 1.11). A nutrient-rich microenvironment is, therefore, created as the cells with different metabolic activities occupy discrete layers, and their metabolic output can be used as a nutrient or energy source for the growth of other residing microorganisms (Davey and O'toole, 2000; López et al., 2010).

Microbial cells within biofilms are optimally organised to make use of all nutrients available, and are also capable of removing any potential toxic metabolites (Davey and O'toole, 2000; Sutherland, 2001; Stoodley et al., 2002b). In addition, biofilms can produce and degrade organic matter and recycle nitrogen, sulphur and many other metals to increase nutrient levels within biofilms (Davey and O'toole, 2000). Nutrient levels within biofilms can be further enhanced by killing sister cells to release the internal contents for other living cells (Branda et al., 2001; González-Pastor et al., 2003). Therefore, the concentration of nutrients in the biofilms can be many times higher than the ambient water (Rao et al., 1997). The nutrient-rich and protected microenvironment inside biofilms appears to be an adaptive strategy for the persistence under unfavourable conditions of different microorganisms as they can position themselves in microniches where they can still propagate (Percival et al., 2000).
Figure 1.10: Cartoon illustrating the importance of cell to cell communication for bacteria and/or other microorganisms in maintaining the three dimensional structure and their multicellular behaviour in biofilms. (Reprint permission obtained from Centre for Biofilm Engineering).

Figure 1.11: The heterogeneity pattern of oxygen depletion within mature biofilms. Bacteria at different levels will use different metabolisms for survival. (Reprint permission obtained from Centre for Biofilm Engineering).
1.5.4 Biofilms in Water Distribution Systems

For the water industry, the presence of biofilms is a double-edged-sword. Biofilms can help to improve water quality by consuming water pollutants such as biodegradable dissolved organic carbon, ammonium nitrogen and ferrous iron for growth purposes (Rittmann, 2004). However, at the same time, biofilms can present significant technical, aesthetic and hygienic problems as they impact on drinking water quality leading to a multitude of industrial problems associated with microbial regrowth and odour problems (Percival et al., 2000; Wingender and Flemming, 2011).

In water distribution systems, the safety and quality of the distributed water depends on the integrity of the distribution system. Any break in that integrity may lead to contamination and cause a variety of organisms to gain access into the distribution system to form biofilms (Figure 1.12) (Percival et al., 2000). However, sometime these pathogens also come from the source water. Furthermore, these biofilms can also harbour a large numbers of opportunistic pathogens (Table 1.1), acting as their environmental reservoir and supporting their growth. To date, it is known that biofilms can support the growth of *Legionella* spp., *Helicobacter pylori*, *Mycobacterium* spp. and *Aeromonas* spp. by sharing the ecological benefits such as protection from various disinfectants and the nutrient-rich microenvironment inside biofilms (Percival et al., 2000; Wingender and Flemming, 2011). The significance of biofilms for microbial ecology has led to the proposition that biofilm growth and species composition should be monitored to a greater degree (Percival and Walker, 1999).
Figure 1.12: The potential for biofilm development in water distribution systems. (Reprint permission obtained from Centre for Biofilm Engineering).

Table 1.1: Major infectious agents associated with biofilms in water distribution systems (Percival et al., 2000).

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Fungi and Yeast</th>
<th>Protozoa</th>
<th>Virus</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudomonas</em> sp</td>
<td><em>Candida parasilosis</em></td>
<td><em>Cryptosporidium</em> sp</td>
<td><em>Hepatitis A</em></td>
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<tr>
<td><em>Flavobacterium</em> sp</td>
<td><em>Rhodotorula rubra</em></td>
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<td><em>Norwalk virus</em></td>
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<tr>
<td><em>Xanthomonas</em> sp</td>
<td><em>Aurebasidium pullulans</em></td>
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<tr>
<td><em>Corynebacterium</em> sp</td>
<td><em>Cryptococcus albidus</em></td>
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<td><em>Nocardia</em> sp</td>
<td><em>Cryptococcus uniguttulatus</em></td>
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<td><em>Enterbacter</em> sp</td>
<td><em>Torulaspora rosei</em></td>
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<td><em>Staphylococcus</em> sp</td>
<td><em>Rhodoturula minuta</em></td>
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<tr>
<td><em>Escherichia coli</em></td>
<td><em>Rhodoturula minita</em></td>
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<td><em>Yersinia</em> sp</td>
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<td><em>Mycobacterium</em> sp</td>
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<td><em>Salmonella</em> sp</td>
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<td><em>Shigella</em> sp</td>
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<td><em>Campylobacter</em> sp</td>
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<td><em>Legionella</em> sp</td>
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<td><em>Aeromonas</em> sp</td>
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<td><em>Vibrio</em> cholerae</td>
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<td><em>Helicobacter</em> pylori</td>
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1.5.5 Aquatic Biofilms and Cryptosporidium

Studies have shown that Cryptosporidium oocysts incorporate readily into biofilms (Rogers and Keevil, 1995; Wolyniak et al., 2009, 2010), and concerns have been expressed over the sudden sloughing of biofilms from water pipes with an accumulated aggregation or ‘bolus’ of oocysts that dramatically increase the infective dose needed to cause infection (Keevil, 2003). In addition, the associations among biofilm communities is believed to influence the propagation of Cryptosporidium through both environmental and water treatment systems (Parsek and Singh, 2003; Searcy et al., 2006). However, the behaviour of Cryptosporidium within a nutrient-rich biofilm is still not well defined (Fisher et al., 2000; Angles et al., 2007).

1.5.6 Hypothesis

It is understood that Cryptosporidium can multiply extracellularly and that they are highly adaptable pathogens, therefore their subsequent fate after being captured into biofilms is worthy of investigation. In addition, previous studies have shown that Cryptosporidium metabolism is extremely streamlined as compared to other coccidian parasites, yet it still has the capability to respire both aerobically and anaerobically, providing it with the flexibility to salvage nutrients from the external environment (Abrahamsen et al., 2004; Barta and Thompson, 2006). Moreover, Cryptosporidium has the ability to take up and catabolise monosugars as well as store, synthesise and catabolise polysaccharides for ATP production via the glycolysis pathway (Abrahamsen et al., 2004). Therefore, this PhD study
hypothesises that Cryptosporidium can multiply extracellularly within the nutrient-rich environment of biofilms.

1.6 References


Rueckert, S., Chantangsi, C. & Leander, B. S. 2010. Molecular systematics of marine gregarines (Apicomplexa) from North-eastern Pacific polychaetes and nemertans, with descriptions of three novel species: *Lecudina phyllochaetopteri* sp. nov., *Difficilina tubulani* sp. nov. and *Difficilina paranemertis* sp. nov. *International Journal of Systematic and Evolutionary Microbiology*, 60, 2681-2690.


2

Optimisation of Quantitative Polymerase Chain Reaction (qPCR) Protocols for the Quantification of *Cryptosporidium* in Aquatic Biofilm Systems
2.1 Introduction

Several cell-free culture studies have shown the importance of quantification data in confirming the possibility of *Cryptosporidium* extracellular multiplication (Zhang *et al.*, 2009; Hijjawi *et al.*, 2010). Therefore, quantitative analysis is the first step to determining the relationship between *Cryptosporidium* and biofilms. To date, many quantitative methods have been used to quantify *Cryptosporidium* from non-pure culture samples such as cell culture (Di Giovanni and Lechevallier, 2005), and waste water (Fontaine and Guillot, 2003a; Guy *et al.*, 2003; Alonso *et al.*, 2011). These techniques include immunofluorescence assays (Siddons *et al.*, 1992; Mayer and Palmer, 1996; Schets *et al.*, 2005), flow cytometry (Vesey *et al.*, 1993; Vesey *et al.*, 1997; Cole *et al.*, 1999; Moss and Arrowood, 2001; Kato and Bowman, 2002; Power *et al.*, 2003; Hsu *et al.*, 2005) and more recently, real-time quantitative polymerase chain reaction (qPCR) (Mayer and Palmer, 1996; Tanriverdi *et al.*, 2002; Fontaine and Guillot, 2003a;b; Guy *et al.*, 2003; Di Giovanni and Lechevallier, 2005; Jothikumar *et al.*, 2008; Alonso *et al.*, 2011).

Quantitative PCR has been shown to be the most sensitive technique among these available methods (Guy *et al.*, 2003; Hadfield *et al.*, 2011). It can detect *Cryptosporidium* at concentrations as low as 10 oocysts (Guy *et al.*, 2003). It quantifies and monitors the increasing amount of PCR product/DNA during the exponential stage of the PCR cycle (Macdonald *et al.*, 2002). The system uses a fluorescent dye/probe to generate a fluorescent signal proportional to the initial amount of template DNA (Heid *et al.*, 1996; Alonso *et al.*, 2011). The threshold cycle number (CT; fractional PCR cycle number) at which a significant increase in target signal fluorescence above the baseline is first detected can be used as a
quantitative method (Di Giovanni and Lechevallier, 2005). The higher the starting copy number of the target nucleic acid, the sooner a significant increase in fluorescence is obtained and a lower CT value will be generated (Heid et al., 1996; Fontaine and Guillot, 2002). The unknown number of microorganisms present in each sample can be determined by obtaining its CT value and estimating the starting copy number of target organism from the standard curves of known quantities of microorganisms (Fontaine and Guillot, 2002).

So far, there has been no standardised qPCR protocol for detecting Cryptosporidium from a mixed sample. Each published protocol has been designed according to the properties of the experimental samples, hence substantial variability was observed in current protocols. It is necessary to design a sensitive qPCR protocol to detect minute amounts of target DNAs in the presence of large background flora. Hence, the aim of this study was to design and optimise a qPCR protocol suitable for detecting Cryptosporidium in biofilm samples.

2.2 Material and Methods

2.2.1 Bacterial Strains and Media

Wild type Pseudomonas aeruginosa bacteria (PA01) were used to establish the biofilms for this study. Before incubating into the biofilm flow cell system, Pseudomonas cultures were maintained on Pseudomonas agar (Beckon-Dickson) at 37°C. A 10% solution of tryptic soy broth (TSB; Beckon-Dickson) was used as the flow-through media in all biofilm experiments.
2.2.2 Cryptosporidium Isolation and Purification

*Cryptosporidium parvum* cattle genotype (Swiss cattle C26) oocysts were obtained from the Institute of Parasitology, University of Zurich and were subsequently passaged through and purified from ARC/Swiss mice as described previously (Meloni and Thompson, 1996). Purified oocysts were stored in sterile 1 x phosphate buffered saline (PBS) with antibiotics (10,000 U penicillin G and 0.01 g/L streptomycin; Sigma) at 4°C before use. Oocysts used in biofilm experiments were less than 4 weeks old and were decontaminated with 2% household bleach at room temperature. Identical batches of oocysts were used for parallel control experiments.

2.2.3 Flow Cell Biofilm Systems

Flow cell biofilm systems were set up as previously described (Werner *et al.*, 2004), except that here, the system was modified to be a fully closed system with no air intake, to prevent air contamination (Figure 2.1). Instead, two capillary flow cells (20 cm in length) were run in parallel, and a silicon tube was attached between the influent and effluent to ensure a smooth air flow. The pump (Cole Parmer 73160-10) used in this study was observed to produce a turbulent flow, therefore no stirrer was included to ensure homogeneity and oocysts were consistently supplied into the flow cells over the course of the experiment. Five-litre glass bottles were used for both influent and effluent media. All experiments were performed at room temperature under sterile and dark environmental conditions.
*P. aeruginosa* inoculum with a turbidity equivalent to that of 1 McFardland standard was prepared from *Pseudomonas* agar and 1 mL of inoculum was transferred to the flow cells as described by Werner *et al.* (2004). To allow bacterial attachment to the surface of the flow tube, no flow was initiated for the first 24 h. Following 24 hours incubation under non-flow conditions, decontaminated oocysts were injected into the influent medium and the flow initiated and continued (60 mL/h) for 1, 3 or 6 days. Biofilms exposed to *Cryptosporidium* oocysts are hereafter referred to as *Cryptosporidium*–exposed biofilm samples.

The volume of influent medium and the number of introduced oocysts were adjusted according to the duration of the experiment (number of days). The experiment was designed such that 5 L of 10% TSB was sufficient for a 3-day experiment. The number of oocysts introduced in the influent medium was calculated so that the biofilms received $1 \times 10^6$ oocysts every 24 h. In order to avoid air contamination, the oocysts were introduced at the beginning of the experiment (for all experiments), and during an influent medium change after three days (for the six day experiment). Two controls were set up simultaneously:

1) No biofilm was developed. Decontaminated oocysts were added into the flow system at similar rates, but without any biofilm established within the flow system. This sample is presented as the biofilm-free control.

2) No oocysts were added. Biofilms were grown in the flow system, without the introduction of *Cryptosporidium* oocysts. This sample is presented as the biofilm-only control.

All experiments utilised two flow tubes simultaneously for each treatment and were repeated three times.
2.2.4 Biofilm Dispersion

Biofilms were dispersed by incubation in 500 nM sodium nitriporusside (Nitric oxide; Sigma Aldrich) overnight, after which the flow cells were gently washed with sterile 1 x PBS several times to completely detach the biofilms from the flow cell surface (Barraud et al., 2009). The cell suspensions were further washed several times with sterile 1 x PBS to remove any residual nitric oxide and immediately post-fixed with 2.5% paraformaldehyde in sterile 1 x PBS for 20 minutes at 4°C. The cells were pelleted at 3500 g for 10 minutes and resuspended in sterile 1 x PBS to a final volume of 400 µL. To quantify the total number of parasites present at the end of each experimental period, the cells in the effluent bottle (dispersed biofilms,
unattached bacteria and oocysts) were also collected by centrifuging at 4°C, at 1000 rpm for 1 hour and resuspended to a final volume of 2 mL.

2.2.5 DNA Extraction

Aliquots of flow cell biofilms (100 µL) and effluent samples (500 µL) were used for DNA extraction. The freeze-thaw DNA extraction method was adopted for all samples. The samples were frozen in liquid nitrogen for 5 minutes and thawed in boiling water. This freeze-thaw process was repeated 12 times. The samples were further purified using the Promega Genomic DNA Extraction Kit. The purification process was performed as described in the manual provided with the kit, except the lysis step was modified from a total of 15 to 45 minutes to allow complete lysing of biofilm aggregates. After the purification process, the DNA was resuspended to a final volume of 50 µL in the DNA suspension buffer provided with the kit.

2.2.6 qPCR Optimisation

The DNA-based technique of quantitative polymerase chain reaction (qPCR) was utilised to quantify the numbers of Cryptosporidium within 1, 3 and 6 day-old biofilms. All qPCR reactions were performed on a Qiagen Rotor Gene 2 system. The acceptable CT cut-off value is 35.
2.2.6.1 Specificity Testing: Primer Sets Testing

As PCR efficiency is mainly dependent on the primer used (Bustin et al., 2009), two primer sets were compared, namely RH primers (Macdonald et al., 2002) that target unknown genes and GAPDH primers that are designed to target the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. The RH primer set was chosen as they were readily available and are often used for in-vitro drug screening by Prof. Andrew Thompson’s lab. The GAPDH primers set was designed as this gene is conserved across the developmental stages and a previous qPCR study by Zhang et al. (2010) had successfully shown that Cryptosporidium can multiply in cell free culture by targeting GAPDH gene. The comparison of primers was made by constructing standard curves using 5 genomic DNA duplicates extracted from a known number of oocysts and serially diluted at 1:10 dilution ratio, calibrated to correspond from $10^0$ to $10^5$ of oocysts. Cryptosporidium DNA was extracted as previously described in section 2.2.5.

1) RH Forward  5’ AAGAAGGGCCGTGTTGGCTTA 3’
   RH Reverse   5’ GGGATTCAGCCCACCAGAAT 3’
2) GAPDH Forward 5’ ATCAAGCCGTAAAGAGCA 3’
   GAPDH Reverse 5’ AAATCGGTCGAGACGACATC 3’

All primer sets were shown to have similar annealing points at 57°C. Hence, similar qPCR conditions were used for each respective primer set. Master mixes were made up using 12.5 µL of Promega GoTaq master mix, 2.5 mM of bovine albumin serum, 10 µM primers specific for C. parvum and 5 µL of DNA to a final volume of 25 µL. Thermal cycling was performed at 95°C for 2 minutes to activate Go-Taq polymerase, the amplification conditions involved 40 cycles of 15 sec at 94°C and 1
minute at 60°C. CT values were achieved from the fixed threshold limit of 0.05. Due to the tendency of pipetting error, each sample was performed in triplicate; the two most closely related CT readings were selected. Negative controls without DNA were included for each reaction.

2.2.6.2 Sensitivity

Sensitivity refers to the minimum number of DNA copies in a sample that can be measured accurately and this is significantly dependent upon the primers used (Bustin et al., 2009). The sensitivity of a qPCR reaction can be determined by the correlation coefficient, $R^2$ that is produced from a standard curve (Bustin et al., 2009). A known amount of oocysts ($2 \times 10^6$) was added into 1 McFarland standard of *Pseudomonas* bacterial culture and referred to as mixed culture. DNA from *Cryptosporidium*, the mixed culture and a 6 day-old *Cryptosporidium*-exposed biofilm were extracted as described in section 2.2.5. Standard curves and qPCR reactions were set up as described in section 2.2.6.1. *Cryptosporidium* oocysts ($10^5$) were used as a positive control and biofilm samples were used as a comparison sample.

2.2.6.3 Specificity

To determine the specificity of each primer set, two different methods were used in this study. Firstly, a BLAST (Basic Local Alignment Search Tool) search was performed using the known primer sequence via the NCBI (National Center for Biotechnology Information) website. Secondly, the specificity of each primer set can
also be determined by qPCR analysis. The Promega Go-Taq master mix uses SYBR
Green as the fluorescent assay that will bind to any double stranded DNA, therefore
melting curve analysis can be performed after the quantification cycle (Arany, 2008)
for primer specificity examination. Melting curve was programmed as the last step of
qPCR amplification cycle of Cryptosporidium, mixed culture and 6 day-old
Cryptosporidium-exposed biofilms. When the melting temperature of the amplicon
is reached, the fluorescence decreases immediately due to the splitting of double
stranded DNA dissociating into single strands (Arany, 2008). The observation of a
single peak indicates that only one target gene is amplified but the observation of
more than one peak indicates that this primer set is non-specific and hence produces
false positive readings of the quantification results (Heid et al., 1996).

2.2.6.4 Specificity to Biofilm-only Sample

DNA from a 6 day-old biofilm-only sample was used to test the specificity of
GAPDH primers. For comparison, $10^5$ oocysts DNA were included. Similar qPCR
master mix, quantification and melting curve settings as described in sections 2.2.6.1
and 2.2.6.3 were used for the study.

2.2.6.5 qPCR Inhibition

Bacteria form biofilms to survive in harsh environments and 99.9% of bacteria grow
in biofilms (Donlan and Costerton, 2002). Therefore, it is expected that biofilms
have an abundance of bacteria within their systems (Isaac and Holloway, 1972). The
high abundance of bacteria may cause qPCR inhibition due to the high
concentrations of template DNA (Innis and Gelfand, 1990). Therefore, to determine whether this would affect the efficacy of primers in quantifying Cryptosporidium, 1 x 10^6 of oocysts were added into 1 McFarland Standard of Pseudomonas culture and their DNA extracted as described above. A standard curve was constructed from a serial of 1:10 dilutions of DNA, calibrated to correspond from 10^0 to 10^5 of oocysts. A set of GAPDH primers were used and a similar qPCR setting as above was applied. Non-template DNA negative control and a 10^5 oocysts DNA positive control were included for comparison.

2.2.6.6 Detection Limit of Biofilm Sample

To ensure a high efficacy of the qPCR reaction, a tenfold serial dilution of 1, 3, and 6 day-old Cryptosporidium–exposed biofilm (flow cell and effluent) DNA was performed to dilute out the presence of inhibitors in each sample. The sample’s DNA was extracted as described in section 2.2.5 and was then serially diluted at 1:10, 1:100 and 1:1000. A qPCR reaction was set up as described in section 2.2.6.1. An undiluted sample (1:0) was included for comparison. Six day-old biofilm-only (effluent; 1:1000 dilution) sample was used as negative control (NTC).

2.3 Results

2.3.1 Sensitivity of RH and GAPDH Primers

Figure 2.2.A shows that the GAPDH primers had an inverse linear relationship between CT value (20 to 34) and concentration of oocysts (1 to 10^5). The R^2 = 0.99
indicates this result is highly accurate. However, when similar qPCR conditions were applied using the RH primers, a non-linear relationship of CT value and oocyst concentration was observed (Figure 2.2.B). Results showed that $10^5$ of oocysts could only be detected at cycle 26 and both $10$ and $10^2$ oocyst concentrations produced similar CT values at cycle 30 (Figure 2.2.B). This implies that this primer set failed to detect oocysts at a low concentration, which also explained why the coefficient correlation was low, with $R^2 = 0.87$. In conclusion, the GAPDH primer set is more sensitive in detecting single oocysts.

![Figure 2.2: Generation of standard curve from the amplification of serially diluted C. parvum oocysts using (A) GAPDH primers and (B) RH primers.](image)
2.3.2 Specificity of RH and GAPDH Primers

BLAST analysis was performed to determine the specificity of the primer sets. No pseudogenes or other unexpected targets were identified using RH or GAPDH primers. Melting curve analysis on a serial tenfold dilution of Cryptosporidium oocysts and a 6 day-old Cryptosporidium-exposed biofilm culture was performed to further confirm these observations. Results (Figure 2.3.A) showed that only GAPDH primers amplified at the correct target site and hence only a single dissociation peak occurred at temperatures between 80°C and 87°C for all samples.

On the other hand, the melting curve analysis of RH primers amplified fragments produced several distinctive dissociation peaks at temperatures between 77°C to 87°C (Figure 2.3.B). Two dissociation peaks at temperatures between 80°C to 85°C and 87°C to 90°C were also observed in 6 day-old Cryptosporidium-exposed biofilm. The origin of these peaks was not investigated. However, the observation of multiple peaks will cause false positive readings in quantification and therefore it was concluded that the GAPDH primer set is more specific for Cryptosporidium than the RH primers.
2.3.3 Biofilm-only Sample

The specificity of the GAPDH primer set was further analysed using biofilm-only samples. A known amount of *Cryptosporidium* oocysts ($10^5$) was used as the positive control. Figure 2.4 shows that unlike the positive control, the biofilm-only samples did not produce quantification and melting curves, which indicated that the
quantification values obtained from Cryptosporidium-exposed biofilms were entirely due to Cryptosporidium only.

Figure 2.4: Amplification (A) and melting curves (B) generated by GAPDH primers from $10^5$ oocysts concentration (red) and 6 day-old biofilm-only samples (black).

2.3.4 qPCR Inhibition - High DNA Template Concentration

In a preliminary comparison (Figure 2.5), it was observed that a high concentration of mixed DNA template affected the qPCR efficacy in quantifying Cryptosporidium. From a serially diluted mixed DNA template, the concentration of $10^5$ oocysts not only produced a similar threshold cycle as $10^4$ oocysts Cryptosporidium but it was also 6 threshold cycles higher than the $10^5$ pure Cryptosporidium oocysts. However, when the average CT values from the amplification of $10^4$, $10^3$, $10^2$, $10^1$ oocysts from the mixed samples were compared to pure oocysts as shown in figure 2.2.A, a
similar threshold cycle was observed. These few observations suggested that a high concentration of mixed DNA template may suppress qPCR efficacy. Hence, to properly quantify Cryptosporidium numbers from biofilm samples, the detection limit of Cryptosporidium needed to be confirmed.
Figure 2.5: Comparison of CT values generated from $10^5$ pure oocysts samples (positive controls) and the tenfold serial dilutions of mixed cultures of Cryptosporidium oocysts ranging from $10^5$-$10^1$ and Pseudomonas bacterial (M) DNA. Table shows the representative colour of each sample and its CT values.
2.3.5 Flow Cell Biofilm Detection Limit

Quantification results showed that 1:0 (undiluted), 1:10 and 1:100 dilutions of 1 day-old *Cryptosporidium* exposed biofilm samples generated CT values ranging from 32 to 38 (Figure 2.6.A). However, the absence of peak from the melting curve analysis (Figure 2.6.B) showed that false positive results were produced from the 1:0 and 1:10 dilutions. As such, it can be concluded from these observations that the detection limit of 1 day-old *Cryptosporidium*-exposed biofilm sample was 1:100 dilution.

The detection limit for 3 day-old *Cryptosporidium*-exposed biofilm samples was also determined to be at a 1:100 dilution (Figure 2.7.A). Furthermore, only one single peak was observed from melting curve analysis indicating that there were no false positive results (Figure 2.7.B). The melting curve analysis also showed that no products were amplified from the undiluted samples (1:0), which coincides with the quantification observation.

Similar observations were also seen for the undiluted (1:0) 6 day-old *Cryptosporidium*-exposed biofilms. No quantification data was obtained (Figure 2.8.A) but melting curve analysis showed multiple unspecific peaks that may have resulted from the impurities generated within mature biofilms (Figure 2.8.B). However, when the biofilm sample was diluted to 1:10, 1:100 and 1:1000 times, *Cryptosporidium* was detected at the threshold cycle of 38, 36 and 33. The observation of a single peak from melting curve analysis further confirmed that only *Cryptosporidium* DNA was amplified from 1:1000 dilution sample during the quantification cycle. Therefore, the detection limit of this sample was 1:1000 dilution.
Figure 2.6: Amplification (A) and melting curves (B) of 1 day-old Cryptosporidium-exposed biofilms (flow cell). Table shows the representative colour of each sample and its CT value.
Figure 2.7: Amplification (A) and melting curves (B) of 3 day-old *Cryptosporidium*-exposed biofilms (flow cell). Table shows the representative colour of each sample and its CT value.
Figure 2.8: Amplification (A) and melting curves (B) of 6 day-old *Cryptosporidium*-exposed biofilms (flow cell). Table shows the representative colour of each sample and its CT value. Arrow indicates unspecific melting curve.
2.3.6 Detection Limit of Effluent Samples

Quantification results showed that Cryptosporidium from 1 day-old Cryptosporidium-exposed biofilms could be detected at undiluted (1:0; CT= ~29), 1:10 (CT= ~33) and 1:100 (CT= ~34) ratios (Figure 2.9.A). The observation of a single peak from the melting curve analysis for each of the dilution showed that only Cryptosporidium parasites were amplified from the sample (Figure 2.9.B). Therefore, current results showed that the 1:100 dilution was the lowest detection limit for this sample.

As the biofilms matured, Cryptosporidium could not be detected from undiluted 3 day-old Cryptosporidium-exposed biofilm effluent (Figure 2.10). Cryptosporidium presence was revealed when dilutions were performed at 1:10 (CT= ~33) and 1:100 (CT = ~34) ratios (Figure 2.10.A). No other non specific peak was observed from the melting curve analysis (Figure 2.10.B). No quantification data was obtained when the sample was further diluted 1:1000 times. Therefore, the lowest detection limit for 3 day-old Cryptosporidium-exposed biofilm sample was 1:100.

The melting curve analysis of 6 day-old Cryptosporidium-exposed biofilm effluent showed that dilutions were needed to decrease false positive quantitative results as multiple peaks were observed from the undiluted sample (1:0; Figure 2.11.B). However, once the sample was diluted 1:10 (CT= ~28), 1:100 (CT= ~30) and 1:1000 (CT= ~33), only a single distinct peak was observed (Figure 2.11). Hence, this demonstrated that the lowest detection limit for this sample was 1:1000.
Figure 2.9: Amplification (A) and melting curves (B) of 1 day-old *Cryptosporidium*-exposed biofilms (effluent). Table shows the representative colour of each sample and its CT value.
Figure 2.10: Amplification (A) and melting curves (B) of 3 day-old Cryptosporidium-exposed biofilms (effluent). Table shows the representative colour of each sample and its CT value.
Figure 2.11: Amplification (A) and melting curves (B) of 6 day-old Cryptosporidium-exposed biofilms (effluent). Table shows the representative colour of each sample and its CT value.
2.4 Discussion

This study focused on optimising qPCR technology to quantify the number of *Cryptosporidium* within a microbial biofilm system and has successfully developed and optimised a qPCR protocol that can detect low quantities of *Cryptosporidium* directly from a biofilm sample. Biofilms are difficult samples to tackle as they contain high concentrations of bacterial DNA (Donlan and Costerton, 2002), matrix (Dominiak *et al.*, 2011; Montanaro *et al.*, 2011), secondary metabolites (Plante *et al.*, 2011), organic and inorganic substances (Guy *et al.*, 2003) and polysaccharides (Narvaez-Zapata *et al.*, 2005) that can act as qPCR inhibitors. Furthermore, unlike other waste water or sewage studies, no *Cryptosporidium* was purified from the biofilm samples through the use of immunomagnetic separation (Lowery *et al.*, 2001; Hallier-Soulier and Guillot, 2003; Jiang *et al.*, 2005; Hashimoto *et al.*, 2006). This is because this technique only targets oocysts and will not pick up other developmental stages and also each additional step of purification may introduce more opportunities for loss. Therefore, it has been suggested that a more crude purification method is preferred to avoid the loss of too much DNA (Plante *et al.*, 2011). Therefore, this study extracted DNA directly from the concentrated biofilm samples, increased the qPCR efficacy by designing a new primer set (GAPDH), and decreased the inhibitory process by dilution. With this, the opportunity for qPCR to also detect other life cycle stages is increased.

In this study, SYBR green was used for quantification and identification of *Cryptosporidium*. Although the Taqman probe has been widely used in detecting *Cryptosporidium* (Fontaine and Guillot, 2002; Macdonald *et al.*, 2002; Guy *et al.*, 2003; Alonso *et al.*, 2011), a previous study has shown no significant differences
between the Taqman and SYBR technologies (Maeda et al., 2003). In addition, the SYBR green assay involves fewer manipulations than the Taqman assay (Kirakodu et al., 2008), hence SYBR green was chosen for this study. To increase the qPCR efficacy within the biofilm sample, two different primers sets were compared: the RH and GAPDH primer pairs. The RH primer set is frequently used in *in-vitro* cultures to assess drug efficacy (Macdonald et al., 2002), but in the current study this primer set was found not to be suitable for detecting *Cryptosporidium* from highly concentrated biofilm samples. In contrast, the newly designed GAPDH primer set was shown to be more suitable for biofilm analysis. When both RH and GAPDH primers were used to quantify a known amount of serially diluted *Cryptosporidium* oocysts, GAPDH primers generated more reproducible results and were more specific and sensitive in quantifying *Cryptosporidium*.

The sensitivity of GAPDH primers was further assessed by introducing known amounts of oocysts into bacterial cultures. It was observed that the CT value of $10^5$ oocysts in this mixture was higher than the pure culture oocysts. This revealed that high concentrations of bacterial DNA imposed an inhibitory effect on qPCR efficacy in quantifying *Cryptosporidium* and a dilution process may be able to decrease this effect. This is because the CT values of other known amounts of oocysts from the mixture were equal to those obtained from pure oocyst cultures. This observation agrees with other studies where the dilution method was very useful in reducing any effect of the inhibitor (Mayer and Palmer, 1996; Plante et al., 2011). Contrary to Dubois et al. (2007), the current results showed that this process did not affect qPCR sensitivity.

The dilution method was also particularly useful when analysing the biofilms samples. As the biofilm matured, multiple melting curve peaks were observed from
the effluent of undiluted 3 and 6 day-old Cryptosporidium-exposed biofilms. This can be due to the abundance of bacterial and Cryptosporidium DNA within the systems. As such, not all DNA were denatured completely during the denaturation cycle (Innis and Gelfand, 1990), hence SYBR green not only bound to the Cryptosporidium double stranded DNA, but also bacterial DNA, which resulted in false positive quantification data. However, the observation of single peaks from the melting curve analysis of the diluted samples showed that qPCR efficacy can be restored by diluting the PCR inhibitors. A previous study supports the present observation that more PCR signals were observed at higher dilutions but not at lower dilutions (Mayer and Palmer, 1996). In the case of samples where there is a high DNA background, it is necessary to evaluate the quantitative limit for each sample type prior to the quantification process, as was undertaken in this study. In addition, except for the 1 day-old Cryptosporidium-exposed biofilms (flow cell), the CT value obtained from the lowest detection limit of the biofilm samples was below cycle 35, indicating the quantification results are reliable. This is because in the study by Nolan et al. (2006), they observed that above 35 cycles, the variability of qPCR is greater and quantification becomes unreliable. Hence, the method developed here will be useful in assessing the presence of Cryptosporidium within biofilm systems.

In summary, a qPCR protocol that is suitable to analyse and quantify Cryptosporidium within a biofilm system was developed. This approach will contribute to the understanding of the fate of Cryptosporidium oocysts after encountering biofilm in the flow cell system. However, this qPCR result cannot provide information about the type of Cryptosporidium stages within biofilms, therefore further experiments such as flow cytometry and microscopy analyses were undertaken (Chapters 3 & 4).
2.5 References


3

Evaluation of Flow Cytometric Analysis for Detecting *Cryptosporidium* in Aquatic Biofilm Systems
3.1 Introduction

Flow cytometry is an automated microscopy process that can rapidly detect and sort 1000 to 25,000 cells sec\(^{-1}\) based on pre-determined optical characteristics (Troussellier et al., 1993; Veal et al., 2000; Davey and Winson, 2003). It is widely used in the food, water and medical industries for bulk scale monitoring of microbial cells (Vesey et al., 1993; Gunasekera et al., 2000; Wang et al., 2010). As it can analyse thousands of cells in a few minutes, this leads to the relatively straightforward acquisition of statistically significant results as compared to conventional microscopy (Davey, 2002; Davey and Winson, 2003). In addition, by analysing many more cells, rare cell types or morphologies are more likely to be detected (Vives-Rego et al., 2000; Davey, 2002). The application of flow cytometry in microbial fields is further expanded by utilising fluorescent stains to discriminate and identify targeted cells from a mixed sample (Collier and Campbell, 1999; Sandt et al., 2007). These fluorescent stains include fluorescent antibodies that selectively label specific types of microorganisms based on the enumeration of surface antigens on the target microorganisms, fluorescent in situ hybridisation, and a wide range of specific and non-specific fluorescent reagents (Davey and Kell, 1996; Collier and Campbell, 1999; Veal et al., 2000). Therefore this allows for multiparametric data acquisition and multivariate data analysis at high speeds (Davey and Kell, 1996).

The flow cytometry technique is less complicated than polymerase chain reaction (Wang et al., 2010). It analyses individual particles or single cells directly by a hydrodynamic focusing process via a flow stream that passes through an excitation laser beam. Interaction between the light beam and the cell causes specific scattering of light and excitation of fluorochromes that can be measured by a photomultiplier
Light scattering is a measurement of optical density that allows the estimation of microbial biomass (Davey and Kell, 1996). In flow cytometry, most of the light scattered is deflected in a forward direction or at small angles and is referred to as forward scatter (Collier and Campbell, 1999). This parameter is often used for cell size measurement. Light that scatters at wider angles is known as side scatter, which is often used to provide information about the internal structure of the cell (Davey and Kell, 1996; Davey, 2002; Hammes and Egli, 2010). This allows for the separation of cells using a gating strategy to target the population of interest in the mixed population. The digitalised data of each particle are then collected and clustered into populations based on their light scatter and fluorescent properties (Hammes and Egli, 2010). This data can be presented as single parameter histograms or as dual parameter dot plots (Veal et al., 2000; Hammes and Egli, 2010).

To date, the application of flow cytometry has been widely used in detecting Cryptosporidium from water and in-vitro cell cultures (Vesey et al., 1991; Vesey et al., 1993; Vesey et al., 1997; Vesey et al., 1998; Neumann et al., 2000; Moss and Arrowood, 2001; Kato and Bowman, 2002; Mele et al., 2003; Hsu et al., 2005; King et al., 2009). However, flow cytometry is not ideal for analysing biofilm samples (Rothemund et al., 1996; Moller et al., 1998; Shapiro, 2003), as flow cytometry is limited to planktonic, single cells and non-aggregated microorganisms (Collier and Campbell, 1999; Hammes and Egli, 2010). Microorganisms within biofilms are difficult to isolate and often appear as aggregated cells (Shapiro, 2003). This increases the difficulty of flow cytometric analysis and underestimates the actual cell count by perceiving aggregated and multiple cells as a single unit.
Although flow cytometry is not suitable for quantifying microorganisms from biofilms, the capability to rapidly discriminate the targeted populations from mixed populations may be used to study the morphological changes of *Cryptosporidium* after exposure to biofilms (Davey and Kell, 1996; Vives-Rego *et al.*, 2000). Since qPCR analysis as previously mentioned in Chapter 2 can only provide quantitative observation, this chapter aimed to evaluate whether flow cytometry can be used to identify various *Cryptosporidium* developmental stages, in order to show that *Cryptosporidium* can multiply in *Pseudomonas* biofilms.

### 3.2 Materials and Methods

#### 3.2.1 *Cryptosporidium parvum* Oocysts and Bacteria

*Cryptosporidium parvum* and bacteria were prepared as described in Chapter 2 (Sections 2.2.2 and 2.2.1).

#### 3.2.2 Biofilm Systems

*Pseudomonas* biofilm systems were set up as described in Chapter 2 (Section 2.2.3). Biofilms were dispersed with nitric oxide and the suspension from both flow cell and effluent were collected and prepared as described in Chapter 2 (Section 2.2.4). Experiments were repeated three times.
3.2.3 Oocysts Decontamination

Known amounts of purified *Cryptosporidium* oocysts were decontaminated with 2% household bleach for 30 minutes at room temperature. Following this, the samples were pelleted at 3500 g for 10 minutes and the supernatant was decanted. The pellet was resuspended to a final volume of 200 µL or 400 µL with sterile 1 x PBS.

3.2.4 *Cryptosporidium* Excystation and Cell-free Culture

Decontaminated oocysts were excysted with acid water (pH 2.5 + 0.5% Trypsin EDTA) for 30 minutes at 37°C. To prepare the cell free culture for the development of other *Cryptosporidium* stages, the following process was performed. The excysted oocysts were pelleted and incubated in 10 mL of maintenance media for 1 h at 37°C. Maintenance media contain 10% RPMI media, 0.03 g/L glutamine, 0.3 g/L sodium bicarbonate, 0.02 g/L bovine bile, 0.1 g/L glucose, 25 µg/L folic acid, 100 µg/L 4-aminobenzoic acid, 50 µg/L calcium pantothenate, 875 µg/L ascorbic acid, 1% foetal calf serum (FCS), 0.36 g/L HEPES buffer, 10,000 U penicillin G and 0.01 g/L streptomycin, adjusted to pH 7.4. The cells were then pelleted at 3500 g for 10 minutes and the supernatant was removed. These cells were resuspended to a final volume of 200 µL in sterile 1 x PBS and was referred to as the “excysted oocysts” sample that contained empty oocysts and various developmental stages.
3.2.5 Flow Cytometry Analysis

Generally, both unexcysted ($10^6$) and excysted oocysts ($10^6$) were used for each flow cytometry experiment in this study. Mixed cultures of bacteria and Cryptosporidium cells were prepared by adding ($10^6$) unexcysted or excysted oocysts into 500 µL of 1 McFarland standard of Pseudomonas bacterial culture. This culture was then pelleted at 3500 g for 10 minutes and fixed with 500 µL of 2.5% paraformaldehyde in sterile 1 x PBS for 20 minutes at 4°C. The samples were subsequently pelleted (3500 g x 10 minutes) and resuspended in 200 µL of sterile 1 x PBS and referred to as cell-free culture sample.

3.2.5.1 Optimisation of Fluorescent Antibodies

Prior to immunolabelling, one million fixed unexcysted oocysts samples (4 tubes) were blocked with 500 µL of 1% rat serum (Sigma) at 37°C. This was done to prevent non-specific antibody binding. The oocysts were then washed with sterile 1 x PBS twice for 5 minutes each and incubated with one of four different concentrations (2.0 µg/mL, 3.5 µg/mL, 5.0 µg/mL, 7.5 µg/mL) of Cy5-Crypt-a-glo™ (Waterborne Inc), a Cryptosporidium oocyst-specific antibody, at room temperature in a dark room for 30 minutes. The cells were then washed twice with 1 x PBS for 5 minutes each. The suspensions were centrifuged at 3500 g for 10 minutes and the supernatant was discarded; the pellet was resuspended in 200 µL of sterile 1 x PBS.

Similarly, FTIC-Sporo-Glo™ (Waterborne Inc), a Cryptosporidium developmental stage-specific antibody was also prepared at different concentrations (2.0 µg/mL, 3.5
µg/mL, 5.0 µg/mL, 7.5 µg/mL) as per the Crypt-a-glo™ antibody, with the exception that excysted oocysts were used. This antibody had previously been shown to label other Cryptosporidium developmental stages (Boxell et al., 2008; Hijjawi et al., 2010). Unexcysted or excysted oocysts without immunolabelling were used as negative controls.

3.2.5.2 Specificity of the Immunofluorescent Antibodies

The specificity of the fluorescent antibodies (5.0 µg/mL of Cy5-Crypt-a-glo™ and 7.5 µg/mL of FTIC-Sporo-Glo™) were examined in the samples containing (i) unexcysted, (ii) excysted oocysts, (iii) 1 McFarland standard of Pseudomonas bacteria, and (iv) mixed cultures of Pseudomonas bacteria and excysted Cryptosporidium oocysts. The immunolabelling process was performed as described in section 3.2.5.1. Unlabelled samples were used as negative controls and as an intensity cut-off point.

3.2.5.3 Comparison between Undiluted and Diluted Cryptosporidium –exposed Biofilm Samples

The presence of a large number of bacteria in biofilms will easily cause flow cytometry to generate an immense wealth of data. Although this might appear to be a positive point, it can obscure the information from low numbers of Cryptosporidium and lead to the wrong interpretation and evaluation. Therefore, it was necessary to determine whether dilutions were needed for the analysis of biofilm samples. Both the biofilms dispersed from flow cells exposed to Cryptosporidium and the
associated effluent (200 µL; 1, 3 or 6 days) were diluted in 1:10 with sterile 1 x PBS. The samples were then labelled with 5.0 µg/mL of Cy5-Crypt-a-glo™ antibody as described in section 3.2.5.1. For comparison, undiluted samples (200 µL) were included and as an intensity cut-off point.

3.2.5.4 Biofilm-only Control

Previous studies had shown that oral biofilms’ matrix trapped and prevented the penetration of antibodies into biofilms (Zhu et al., 2001; Thurnheer et al., 2003). Their observations suggested that the matrix present in this study may trap some antibodies during the immunolabelling process. Since the mature biofilms contained the highest abundance of matrix, 6 day-old biofilm-only samples were labelled with Cy5-Crypt-a-glo™ antibody (5.0 µg/mL) as described in section 3.2.5.1 and unlabelled 6 day-old biofilm-only sample was used as the negative control and as an intensity cut-off point.

3.2.5.5 Flow Cytometry Setting

The optical characteristics of prepared samples (unexcysted and excysted oocysts; undiluted and diluted Cryptosporidium–exposed biofilm samples; biofilm-only control) were analysed on a flow cytometer (FACSCalibur™, BD Biosciences, Sydney). Logarithmic signals were used for all parameters and the forward light scatter detector was set at E00 for all assays. To analyse FTIC-Sporo-Glo™ labelled samples, the forward scatter detector (FSC) was set to 396 V and the green
fluorescent detector set to 467 V. For Cy5-Crypt-a-glo™ labelled samples, the
forward scatter detector was set to 396 V and the red fluorescent detector to 597 V.
For the optimisation experiment (Section 3.2.5.1), the fluorescence signal intensities
of different antibody concentrations (Cy5-Crypt-a-glo™/ FTIC-Sporo-Glo™) were
compared and illustrated using histograms (number of counts vs fluorescent
intensity). A peak was generated by each antibody at a specific wavelength and the
optimal antibody concentration was determined from the sample that generated the
greatest fluorescence intensity.

The optimal antibody concentration and gating was then used to discriminate
Cryptosporidium populations from mixed populations. The acquisition gate was
determined based on the fluorescence (intensity) and forward scatter characteristics
(cell size <μm>, expressed in logarithm number) of labelled pure bacteria and
Cryptosporidium oocysts (unexcysted/excysted). These defined areas were then used
to determine where Cryptosporidium and bacteria would be found in biofilm or
mixed (bacterial and excysted oocysts) samples. All comparisons were made against
negative controls (intensity cut-off point) and the assays were analysed in triplicate.

3.2.6 Confocal Microscopy Analysis

To determine the specificity of Cy5-Crypt-a-glo™ and FITC-Sporo-Glo™
antibodies, excysted oocysts (1 x 10⁶) were immunolabeled with both FTIC-Sporo-
Glo™ and Cy5-Crypt-a-glo™ as previously described in section 3.2.5.1. They were
examined under the Leica SP2 confocal microscope at two different
excitation/emission wavelengths: 488/525 nm (FITC) and 649/688 nm (Cy5).
3.2.7 Scanning Electron Microscopy

When unexcysted oocysts were analysed using flow cytometry, two distinctive subpopulations were observed. As these oocysts were extracted directly from mice gut, it was believed these two subpopulations may have been formed by thin- and thick-walled oocysts. To confirm this possibility, these two subpopulations were sorted by cell sorting and their morphologies examined using SEM. The cells were sorted Dr. Kathy Heel (UWA) using the protocol developed by the Centre of Microscopy, Characterisation and Analysis Centre (UWA). Edwards et al.(2012) showed that thin-walled oocysts have an electron transparent wall allowing for the visualisation of the internal sporozoites under SEM, while no sporozoites could be observed in thick-walled oocysts. As such, an aliquot of cell sorted suspension from both subpopulations were analysed under SEM as described in Chapter 4 (Sections 4.2.8.3 & 4.2.8.4.).

3.3 Results

3.3.1 Confocal Microscopy Analysis

Prior to flow cytometry analysis, populations of excysted oocysts that had been labelled with both Cy5-Crypt-a-glo™ and FTIC-Sporo-Glo™ antibodies were observed under the confocal microscope. Figure 3.1 shows that FTIC-Sporo-Glo™ antibody labelled both oocysts and internal sporozoites while Cy5-Crypt-a-glo™ antibody only labelled oocysts. This showed that Cy5-Crypt-a-glo™ antibody did not label sporozoites.
Figure 3.1: Images of immuno-fluorescently stained excysted oocysts from the same location but at different wavelengths, 488 nm (A: FITC-Sporo-Glo™) and 649 nm (B: Cy5-Crypt-a-glo™). Image C shows bright field image. Yellow arrow indicates sporozoites and white arrows indicate oocysts. Scale bar: 5 µm.

3.3.2 Optimisation of Fluorescent Antibodies

As the concentration of Cryptosporidium oocyst specific antibody Crypt-a-glo™ increased, an increase in fluorescence intensity was identified (Figure 3.2.A). However, it was observed that the fluorescence intensity range produced from 3.5 µg/mL, 5.0 µg/mL, and 7.5 µg/mL were overlapping to one another. Hence, 5.0 µg/mL of Crypt-a-glo™ was therefore considered the optimal for additional experiments (Figure 3.2.A).

A similar optimisation process was also carried out for Cryptosporidium developmental stage-specific antibody, Sporo-Glo™ (Figure 3.2.A). Unlike Crypt-a-glo™ antibody, with the observation of overlapping peaks from different concentrations, for Sporo-Glo™ antibody a similar fluorescent intensity range was generated (Figure 3.2.B). When compared to the negative control, only a small increase in fluorescence intensity was produced from all the labelled excysted oocysts. However, it appeared that 7.5 µg/mL antibody produced a wider range of
fluorescent intensity that could be discriminated within a mixed sample. Hence, the optimal Sporo-Glo™ antibody concentration was determined to be 7.5 µg/mL.

![Figure 3.2: Histogram illustrating fluorescence signal intensities generated from different concentration (2.0 µg/mL, 3.5 µg/mL, 5.0 µg/mL, 7.5 µg/mL) of oocyst specific antibody Crypt-a-glo™ and developmental stages specific antibody, Sporo-Glo™.](image)

3.3.3 The Specificity of Crypt-a-glo™ Antibody

After antibody optimisation, the specificity of Crypt-a-glo™ was determined by comparing labelled unexcysted oocyst samples to the negative control and excysted oocysts samples. No fluorescent intensity was detected from Cryptosporidium negative controls (Figure 3.3.A). Unexcysted and excysted oocysts labelled with Crypt-a-glo™ antibody were clearly separated from background noise and mixed.
population (Figure 3.3.B & C). In addition, no other fluorescently labelled bacterial populations were identified (Figure 3.4.B). This shows that Crypt-a-glo\textsuperscript{TM} antibody is specific to the \textit{Cryptosporidium} oocyst wall (intact/excysted). Results also showed that the highly fluorescent oocysts could be distinctively discriminated within the mixed culture (Figure 3.4.D). This indicated that this measurement could be reliably used to identify and discriminate \textit{Cryptosporidium} from the biofilm using flow cytometry analysis.

Figure 3.3: Comparison of scatter plots (Cy5 intensity vs FSC) of Crypt-a-glo\textsuperscript{TM} labelled unexcysted (B) and excysted \textit{Cryptosporidium} oocysts (C). Unexcysted oocysts (A: negative control) from stock solution were used as comparison to the labelled samples.
3.3.4 The Specificity of Sporo-Glo™ Antibody

Two distinct populations were generated from the excysted oocysts sample, the oocysts and a mixed population, which was possibly formed by various developmental stages (Figure 3.5.C). Although the type of developmental stages
present in the mixed population was unable to be determined, the fluorescent signal of excysted oocysts was shown to be higher than unexcysted oocysts (Figure 3.5.B & C). In addition, Sporo-Glo™ antibody was shown to be specific to *Cryptosporidium*, as a similar bacterial population distribution was generated from both labelled and unlabelled bacteria cultures (Figure 3.6.A & B). However, it was not possible to discriminate the weakly fluorescent signal of Sporo-Glo™ labelled excysted oocyst samples from the mixed culture (Figure 3.6.D). These observations suggested that the poor discriminating capability of Sporo-Glo™ antibody was not suitable for the analysis of *Cryptosporidium* by flow cytometry. As such, this antibody was not used to detect *Cryptosporidium* within biofilms by flow cytometry.
Figure 3.5: Comparison of scatter plots (FITC-intensity vs FSC) of FITC-Sporo-Glo™ labelled unexcysted (B) and excysted Cryptosporidium oocysts (C). Unexcysted oocysts from stock solution (A: Negative controls) were used for comparison to the labelled controls.
Figure 3.6: Comparison of scatter plots (FITC-intensity vs FSC) of FITC-Sporo-Glo™ labelled bacterial (B) and mixed culture of bacterial and excysted Cryptosporidium oocysts (D). Unlabelled bacterial (A: Negative control) and mixed cultures (C: Negative control) were used as comparison to the labelled samples.

3.3.5 The Identity of Oocyst Populations

The discriminating power of Crypt-a-glo™ antibody highlights the potential of using it to track the fate of oocysts morphologically by flow cytometry. In this study, several oocyst populations were frequently observed from both unexcysted and
excysted oocyst controls. These populations were assigned as intact (Figure 3.7) and excysted (Figure 3.9) populations.

3.3.5.1 Intact Oocyst Population - Subpopulations A and B

Intact oocyst populations consisted of unexcysted oocysts that had cell sizes above 4 µm. Within this population, three different cell size subpopulations, A (4 µm) and B (4.5 µm), and C (>4.5 µm) were frequently observed (Figure 3.7). From both unexcysted and excysted oocysts samples, intact oocyst subpopulations A and B were formed by single intact oocysts and it was hypothesized that the differences in cell size was due to the formation of thin and thick-walled oocysts. Hence, cell sorting and SEM were carried out to confirm the identity of these two subpopulations (Figure 3.8). From the SEM results, it was observed that the intact oocyst cell sizes from both subpopulations were well correlated to the flow cytometry observation. However, the differences in oocyst cell sizes were not related to the oocyst wall thickness as both thin and thick-walled oocysts were identified within both subpopulations (Figure 3.8).
Figure 3.7: Subpopulations A, B, and C within intact oocyst population.
3.3.5.2 Intact Oocyst Population - Subpopulation C

Intact oocyst subpopulation C was more frequently observed from the unexcysted oocyst samples. This population was assigned into intact oocyst populations because it had the largest total cell size (> 4.5 µm) and also expressed the highest fluorescent intensity among the oocyst populations (Figure 3.7). Furthermore, previous study (Kar et al., 2011) also showed that unexcysted oocysts tend to aggregate; hence this
population was believed to be formed by many aggregated oocysts that had not excysted.

3.3.5.3 Excysted Oocyst Populations

This study consistently identified excysted oocyst populations from excysted samples only. The fluorescence intensity of this population was similar to the intact oocysts population (Figure 3.9). The excystation process and subsequent release of sporozoites into the external environment deformed oocyst morphology, resulting in variable oocysts sizes within this population.

![Figure 3.9](image)

**Figure 3.9:** Example of excysted oocyst, intact oocysts and developmental stages populations identified from the scatter plot analysis of Crypt-a-glo<sup>TM</sup> labelled excysted oocysts samples.
3.3.6 Biofilm Samples

3.3.6.1 Flow Cell Biofilms

From all the *Cryptosporidium*-exposed biofilm samples (1, 3 and 6 day-old), no distinctive oocyst population was observed in either undiluted or diluted samples (Figure 3.10). This indicated that flow cytometry is not sensitive enough to detect low numbers of *Cryptosporidium* within flow cell biofilms.

3.3.6.2 Effluent Samples

Distinctive oocyst populations were observed from undiluted samples (Figure 3.11.A) but no distinctive oocyst population was observed by flow cytometry from the diluted samples of 1 day-old *Cryptosporidium*-exposed biofilm. Similar observations were also made for 3 and 6 day-old *Cryptosporidium*-exposed biofilms. Undiluted samples revealed 3 distinct oocyst populations from both samples (Figures 3.11C & E) while only 1 or 2 distinct oocyst populations were found from diluted samples (Figures 3.11D & F). It can be concluded from the observations that the dilution process was not required for effluent samples.

3.3.6.3 Biofilms-only Control

Flow cytometry analysis was performed on the undiluted 6 day-old biofilm only control. A fluorescent population in the 2-3-µm size range was observed in the Crypt-a-Glo™ labelled biofilm-only control sample (Figure 3.12.B). As this
population was not identified in pure bacterial culture (Figure 3.12.A) and also no Cryptosporidium was present in 6 day-old biofilm-only controls, it was suspected that it was suspected that Crypt-a-Glo™ may bind to some unknown component of biofilm.

Figure 3.10: Comparison of scatter plots (Cy5-intensity vs FSC) between undiluted and diluted (1:10) Cryptosporidium-exposed biofilm samples (flow cell). All samples were immuno-fluorescently labelled with Crypt-a-glo™ antibody. (A) Undiluted 1 day-old Cryptosporidium-exposed biofilms. (B) Diluted 1 day-old Cryptosporidium-exposed biofilms. (C) Undiluted 3 day-old Cryptosporidium-exposed biofilms. (D) Diluted 3 day-old Cryptosporidium-exposed biofilms. (E) Undiluted 6 day-old Cryptosporidium-exposed biofilms. (F) Diluted 6 day-old Cryptosporidium-exposed biofilms.
Figure 3.11: Comparison of scatter plots (Cy5-intensity vs FSC) between undiluted and diluted (1:10) Cryptosporidium-exposed biofilm samples (effluent). All samples were immuno-fluorescently labelled with Crypt-a-glo™ antibody. (A) Undiluted 1 day-old Cryptosporidium-exposed biofilms. (B) Diluted 1 day-old Cryptosporidium-exposed biofilms. (C) Undiluted 3 day-old Cryptosporidium-exposed biofilms. (D) Diluted 3 day-old Cryptosporidium-exposed biofilms. (E) Undiluted 6 day-old Cryptosporidium-exposed biofilms. (F) Diluted 6 day-old Cryptosporidium-exposed biofilms.
3.4 Discussion

The present study showed the potential of utilising flow cytometry to discriminate *Cryptosporidium* oocyst populations within biofilms. The intense fluorescent expression of Crypt-a-glo™ antibody in labelling *Cryptosporidium* oocysts has successfully allowed for the discrimination of several distinct *Cryptosporidium* oocyst populations from both mixed culture (bacterial and *Cryptosporidium*) and effluents samples. This confirms the use of immunolabelling and flow cytometry cell sizing measurements method as reliable and indicates that it can be used to determine the fate of *Cryptosporidium* oocysts in the biofilm system.

In general, commercial *Cryptosporidium* oocyst antibody binds to the surface antigens of *Cryptosporidium* oocysts walls (Davey and Kell, 1996). Therefore by using Crypt-a-glo™ antibody and the amount of light scattered at a forward angle...
(FSC), discrimination between the two distinctive oocyst populations, intact, and excysted oocysts, from both the unexcysted and excysted cultures were able to be analysed by flow cytometry. Within intact oocyst populations, two single intact subpopulations were constantly observed and SEM showed that the oocyst size differences were not related to oocyst wall thickness. However, as oocyst stocks used in this study were directly extracted from mice intestine, these two oocyst subpopulations may result from still-developing oocysts (small) and well developed oocysts complete with internal sporozoites (large). Hence, these two single, intact oocysts subpopulations were frequently observed from both unexcysted and excysted oocysts cultures.

Furthermore, the finding of Jenkins et al. (2011) supports the present results that aggregated oocyst subpopulations were always present within unexcysted oocyst samples. This is because oocysts in suspension often have an uneven distribution, causing them to be displayed as single or aggregated oocysts (Reynolds et al., 1999). Due to the aggregation of oocysts, flow cytometry identified the total cell sizes within this population to be above 4.5 µm and had the highest fluorescent intensity among oocysts populations. In theory, Cryptosporidium oocysts aggregates should be larger than 8 µm in size, but was never observed in this study. This is most likely due to the orientation of Cryptosporidium aggregates when passing through the flow cytometry detector. Furthermore, this population was not identified from the excysted oocyst oocysts and it is possible that this may be due to these oocysts being exposed to the acidic environment, causing the macromolecules on the oocyst surface to be altered and to reduce aggregation. Therefore, after the excystation process, unexcysted oocysts remained as single entities in the maintenance media.
while oocysts that had been excysted underwent a series of morphological changes, hence the “excysted” oocyst population was also detected.

Due to low number of Cryptosporidium oocysts in flow cell biofilms, no oocyst population was observed in flow cell biofilm. This is because populations observed from flow cytometry often require high densities (10,000 to 500,000 cells) of specific single cells (Davey, 2002). However, two main oocyst populations (intact and excysted) were successfully detected from undiluted effluent samples, showing that dilutions of effluent samples were not necessary for flow cytometry analysis. Although a study by Valdez et al. (1997) demonstrated that the dilution process helped them to eliminate large debris that may clog the instrument and thus were able to reveal more cells by flow cytometry. The present study failed to observe multiple distinctive oocyst populations from diluted biofilm samples. This is because the dilution process not only excluded a large number of bacteria cell data from being collected, but may have also diluted out the cell population of interest. Since this study did not intend to use flow cytometry as a quantification tool, undiluted samples may be the best option for this analysis. Therefore, similar to other studies (Collier and Campbell, 1999; Davey, 2002), the current study determined that flow cytometry was more likely to reveal populations that persist at higher cell densities.

The determination of the type of developmental stages present within the mixed or pure excysted culture using the Sporo-Glo™ antibody failed. Two factors appear to have contributed to this. Firstly, the natural properties of the Sporo-Glo™ antibody. A previous study proposed that for flow cytometric analysis, the characteristics of potential fluorescent stains must be determined (Davey and Kell, 1996). Therefore, to clearly distinguish labelled Cryptosporidium developmental stages from other bacteria or unlabelled cells, Sporo-Glo™ antibody at different concentrations was
tested. Even when used at the highest concentration, Sporo-Glo™ antibody was still unable to identify *Cryptosporidium* developmental stages within mixed cultures by flow cytometry. This could have been caused by the presence of less surface antigens from those extracellular developmental stages that had been growth from a host-devoid system. Sporo-Glo™ antibodies were raised from antigens generated from rats, hence *Cryptosporidium* developmental stages may express more Sporo-Glo™ antigens in *in-vitro* culture and *in-vivo* as compared to cell-free cultures. The low amount of surface antigens may have restricted flow cytometry ability to detect *Cryptosporidium* in mixed population of bacteria and *Cryptosporidium*. Further explanation is difficult, as the actual antigens that the antibody binds to are unknown. Although the observation of increased fluorescence signal after the oocysts been excysted suggested certain surface antigens were expressed during the excystation process (Edwards *et al.*, 2012), the amount of surface antigens were not sufficient to be detected by flow cytometry in the mixed sample. Thus, to allow examination of *Cryptosporidium* extracellular developmental stages in the mixed sample of *Cryptosporidium* and bacteria by flow cytometry, an antibody that is raised against antigens generated from *Cryptosporidium* cell-free cultures is required.

The low number of *Cryptosporidium* developmental stages such as sporozoites, trophozoites, merozoites and macrogamonts from the mixed and cell-free culture may be the second factor contributing to the failure in observing distinct developmental stages from the pure and mixed cultures. Although flow cytometry is 100 to 1000-fold sensitive than traditional microscopic methods (Davey and Kell, 1996), it may still not be sensitive enough to detect low *Cryptosporidium* numbers among a large population of other cells as large number of *Cryptosporidium* are
often required for flow cytometry analysis (Kato and Bowman, 2002). Furthermore, flow cytometry is designed for the analysis of cells in size range of 1-100 µm (Collier and Campbell, 1999) that may also pose some limitations for certain life stages of Cryptosporidium such as microgametes and merozoites that have sizes less than 1 µm (Borowski et al., 2008). Therefore, the present observations agree with previous studies that it is best to identify Cryptosporidium developmental stages within biofilms microscopically (Collier and Campbell, 1999; Shapiro, 2003).

In conclusion, this study confirmed that the fate of oocysts after exposure to biofilms can be tracked morphologically by using fluorescence based flow cytometric analysis methods. The information obtained may be used to determine whether Cryptosporidium can excyst within biofilms. However, the subsequent development of sporozoites will need to be determined by confocal and scanning/transmission electron microscopy.

3.5 References


4

Optimisation of Microscopy Techniques to Reveal the relationship of *Cryptosporidium* with Aquatic Biofilms
4.1 Introduction

Both Chapters 2 and 3 showed that qPCR and flow cytometry cannot provide structural information on the developmental life stages of Cryptosporidium in biofilm systems. Without visual information about the association of Cryptosporidium with biofilms, the morphology of Cryptosporidium in the aquatic biofilms will remain poorly understood. As such, traditional methods of microscopy and structural characterisation may be useful in revealing the subsequent fate of Cryptosporidium after exposure to biofilms. Although it may be time consuming, several studies have shown that microscopy may be the best option in studying morphological changes in microorganisms residing within biofilms (Rothemund et al., 1996; Shapiro, 2003; Muller et al., 2011). A number of microscopy techniques are available for biofilm research. For example confocal laser scanning microscopy, two-photon laser scanning microscopy, blink microscopy, photoactivated localisation microscopy, stochastic optical reconstruction microscopy, direct stochastic optical reconstruction microscopy and structural illumination microscopy have all been used (Jensen and Tolker-Nielsen, 2011). However, not all of these methods are commonly available to researchers, and within Western Australia, confocal microscopy and scanning electron microscopy are the most widely available, and as such, are most suitable for this project for observing Cryptosporidium within biofilms.

Confocal microscopy is one of the most common methods used to study the physiological heterogeneity in biofilms (Palmer and Sternberg, 1999). It provides a non-destructive observational method by labelling all living cells with fluorescent stains (Ghiorse et al., 1996). Confocal microscopy is able to provide high resolution
imaging by combining a thin plane of focus and laser light that creates an intense and
deeper penetrating excitation energy that is detected by photomultiplier tubes
(Palmer and Sternberg, 1999; Pamp et al., 2009). Scanning electron microscopy on
the other hand is one of the most powerful techniques for studying detailed surface
structures at higher resolution (Asahina et al., 2012). It uses electrons for
illumination and electromagnetic lenses for detection and is able to resolve features
as small as 6 nm (Judith, 1982), and hence in addition to confocal microscopy, many
studies have also adopted this technique to study *Cryptosporidium* life stages at
greater resolutions (Valigurová et al., 2008; Borowski et al., 2010; Edwards et al.,
2012).

By using a combination of these two techniques, labelled antibodies specific to
*Cryptosporidium* oocysts and different developmental stages can also be applied to
further confirm observations of *Cryptosporidium* life stages. Therefore, the aim of
this study was to develop and optimise techniques and methodologies to reveal the
interaction between *Cryptosporidium* and biofilms, with particular focus upon
immunolabelling, confocal microscopy and scanning electron microscopy.

### 4.2 Materials and Methods

#### 4.2.1 *Cryptosporidium* Oocysts

*Cryptosporidium parvum* oocysts purification was performed as previously
described in Chapter 2 (Section 2.2.2).
4.2.2 Oocysts Excystation

Known amounts of oocysts required for excystation were decontaminated and excysted as described in Chapter 3 (Sections 3.2.3 and 3.2.4).

4.2.3 Biofilm Systems

The biofilm flow cell system was used as described in Chapter 2 (Section 2.2.3). Three replicates were performed.

4.2.4 Bacterial Preparation

Two different bacteria species were used for biofilm development, *Salmonella typhimurium* and *Pseudomonas aeruginosa*. As *S. typhimurium* was available at the very start of the project, this strain was initially used to test the suitability of the flow cell system in performing optical live imaging. This bacterium was constantly maintained on XLD agar (Becton Dickson) at 37°C. As *Pseudomonas aeruginosa* PA01 is always referred to as model biofilm, therefore, after receiving *Pseudomonas aeruginosa* (PA01) cultures from Dr. Peter Gerling (Murdoch University), this culture was subsequently used to generate biofilms for all of the remaining studies. *Pseudomonas* was maintained on *Pseudomonas* agar (Becton Dickson) at 37°C.
4.2.5 Optical Microscopy (Biofilm Live Imaging)

The first attempt at optical flow cell biofilm live imaging was performed using *Salmonella* (1 McFarland standard). The flow was initiated for 3 or 5 days, and flow cells were visualised directly using an Olympus CX151 microscope.

4.2.6 Confocal Microscopy

4.2.6.1 Live Imaging (Pseudomonas Biofilm-only)

Due to the inability of optical microscopes to resolve the internal structure of semi-transparent or opaque structures of biofilm, live confocal imaging was performed using *Pseudomonas* biofilm. Film Tracer calcein red orange (Invitrogen) was used to stain the biofilms. According to the commercial product sheet, FilmTracer™ calcein red orange is a fluorogenic esterase substrate that passively diffuses into living cells and is usually used as a bacterial activity indicator. Although FilmTracer™ calcein red orange is fluorescent prior to cleavage, the intracellular fluorescence is much brighter than background fluorescence. A similar flow cell system without *Cryptosporidium* as previously described in Chapter 2 (Section 2.2.3) was used. The flow cells were run for 1, 3 or 6 days. At the end of the respective flow periods, the solution containing FilmTracer™ calcein red-orange as described above was transferred into the flow cells using a peristaltic pump at 60 mL/h and the flow was stopped once it was determined that the flow cell was full of the stains. The biofilms in the flow cell were incubated with FilmTracer™ calcein red orange for 1 h in the dark and then washed with sterile distilled water to remove any stain that remained
unbound. The flow cells were observed immediately using Leica SP2 confocal laser scanning microscopy at the FilmTracer™ calcein red orange laser excitation/emission wavelength of 576/590 nm. Average biofilm thickness was determined from three regions - the left, right and middle area - of the flow cell. Serial sections on the xy plane were obtained at 0.44 µm (1 day-old biofilm) and 2-10 µm intervals (3 and 6 day-old biofilms) along the z-axis, and the z-stack images were then analysed with COMSTAT II (Heydorn et al., 2000). Statistical analysis was performed using GraphPad Prism, version 5 (GraphPad Software, San Diego, CA). One-way ANOVA analysis of variance was performed to determine whether there were significant differences in (i) thickness between 1, 3 and 6 day-old biofilm-only controls. This experiment was repeated three times.

4.2.6.2 Specificity of Biofilm Stain

Prior to introducing oocysts into the flow cell system, the specificity of the biofilm stain, FilmTracer™ calcein red orange was examined using mixed-cultures of Pseudomonas bacteria (1 McFarland Standard) and Cryptosporidium oocysts (1 x10⁶). The mixed cultures were pelleted at 3500 g for 10 minutes and incubated with 30 nM of FilmTracer™ calcein red orange in a dark room at room temperature for 1 h. After this, the cells were re-pelleted at 3500 g for 10 minutes and resuspended in 200 µL of sterile 1 x PBS. Confocal images of biofilms were acquired from the flow cells directly using 576 nm and 590 nm laser excitation/emission wavelengths.
4.2.6.3 The Stability of Crypto-Cel Antibody

As FITC-Crypto-Cel (Cellabs) oocyst-specific antibody was available at the very start of the project, it was used to locate oocyst populations within biofilms. However, the stability of this antibody in 10% tryptic soy broth (TSB) for over a 6 day period needed to be determined. To do so, three separate tubes containing $10^6$ oocysts were pelleted at 3500 g for 10 minutes and resuspended in 200 µL of sterile 1 x PBS with rat bovine serum albumin (BSA; Sigma). Ten microlitre of the commercial stock of Crypto-Cel antibody was added into each tube and incubated in the dark room at room temperature for 1 h. After this, the oocysts were washed twice with sterile 1 x PBS for 5 minutes each. They were then pelleted at 3500 g for 10 minutes and incubated in 15 mL of 10% TSB for 1, 3 or 6 days. After the respective incubation periods, the oocysts were washed twice with sterile 1 x PBS for 5 minutes and pelleted at 3500 g for 10 minutes and resuspended to final volume of 200 µL in sterile 1 x PBS. An aliquot of the suspension was analysed directly by confocal microscopy using the excitation and emission wavelength of FITC at 488/525 nm. Both confocal and bright field images were acquired simultaneously.

4.2.6.4 The Specificity of FITC-Sporo-Glo™ Antibody

Both biofilm stain and FITC-Sporo-Glo™ (Waterborne Inc) antibody were introduced directly into living biofilms at the same time, thus, it was important to ensure that the fluorescent emission of both stains would not interfere with one another. As such, one million excysted oocysts were pelleted as described above and resuspended with 470 µL of 30 nM FilmTracer™ calcein red orange stain and 7.5 µg/mL of FITC-Sporo-Glo™ antibody in the dark at room temperature for 1 h. The
oocysts were then washed twice with sterile 1 x PBS and resuspended to a final volume of 200 µL. The sample was further examined using confocal microscopy at two different excitation and emission wavelengths, 488/525 nm (FITC-Sporo-Glo™) and 576/590 nm (FilmTracer™ calcein red orange).

4.2.6.5 Live Imaging (Cryptosporidium-exposed Biofilms)

Similar Cryptosporidium-exposed biofilms systems were set up as described in Chapter 2 (Section 2.2.3), except that the oocysts were pre-stained with Crypto-Cel antibody as described in section 4.2.6.3. The flow cells were run for 1, 3 or 6 days. At the end of the respective flow periods, the solution containing 200 µL of FITC-Sporo-Glo™ antibody and 100 µL of working stock solution of FilmTracer™ calcein red orange was then transferred into flow cells using a peristaltic pump at 60 mL/h. The flow was stopped once it was determined that the flow cell was full of antibody/stains. The flow cells were incubated, washed and examined using confocal microscopy as described above. Two different wavelengths were used to image the respective stains, 488/525 nm (Crypto-Cel and FITC-Sporo-Glo™) and 576/590 nm (FilmTracer™ calcein red orange).

4.2.6.6 Biofilm Dispersion

Biofilms were dispersed and the suspension collected and fixed as described in Chapter 2 (Section 2.2.4). Immunolabelling of Cryptosporidium within biofilms was subsequently performed on these fixed, dispersed biofilms.
4.2.7 Immunolabelling

4.2.7.1 FITC-Sporo-Glo™ Antibody Binding Pattern

Several studies have shown that Sporo-Glo™ can bind to a wide range of Cryptosporidium life stages (Boxell et al., 2008; Hijjawi et al., 2010; Edwards et al., 2012). To further confirm their observations (Boxell et al., 2008; Hijjawi et al., 2010; Edwards et al., 2012), the labelling pattern of FITC-Sporo-Glo™ antibody was investigated. Bright field/transmitted light images were used to determine the nature of oocysts. To maintain Cryptosporidium morphology, 10⁶ unexcysted and excysted oocysts were fixed with 500 µL of 2.5% paraformaldehyde in sterile 1 x PBS for 20 minutes at 4°C. They were subsequently pelleted and labelled with FITC-Sporo-Glo™ antibody as described in Chapter 3 (Section 3.2.5.1). The samples were then examined under confocal microscopy at FITC wavelengths (488/525 nm).

The binding pattern of FITC-Sporo-Glo™ antibody to other Cryptosporidium developmental stages was also examined. Cell-free culture was performed using the excysted oocysts that were incubated in maintenance media for 4 days as described in Chapter 3 (Section 3.2.4).

4.2.7.2 Quantum dot-Sporo-Glo™ Antibody Binding Patterns

Due to very rapid photobleaching of FITC-Sporo-Glo™ antibody when imaging, quantum dot labelled Sporo-Glo™ was used instead, as quantum dots (QDs) do not suffer photobleaching effects. QD 655 anti-rat secondary antibody was purchased from Invitrogen (catalogue no Q-11621MP) and conjugated to Sporo-Glo™
polyclonal primary antibody (Waterborne Inc). The binding pattern of QD- Sporo-Glo™ was examined using both unexcysted and excysted oocysts. The specificity of QD labelling was also performed on 3 day-old Cryptosporidium-exposed biofilms. The cells were pelleted and incubated with 200 µL of blocking buffer (6% BSA + 10% rat serum in sterile 1 x PBS) for 1 h at room temperature. The cells were then washed with sterile 1 x PBS (pH 7.4, 2 times, 5 minutes) and incubated with primary antibody (1:100 in blocking buffers) for 2 h at 37°C. The samples were then washed again with sterile 1 x PBS (2 times, 5 minutes) and incubated with QD 655 anti-rat secondary antibody. Primary antibody was bound with 40 nM QD secondary antibody that had been prepared with incubation buffer (6% BSA in sterile 1 x PBS) for 2 h. The cells were then rinsed 3 times with sterile 1 x PBS and resuspended to a final volume of 100 µL. As QDs have long excitation wavelengths, three different wavelengths (488/525 nm, 576/590 nm and 625/700 nm) were used for examining excysted oocyst samples. The 488 nm wavelength provided the highest fluorescent intensity and was subsequently used to observe all QD labelled samples. Experimental controls included for both Cryptosporidium and 3 day-old Cryptosporidium-exposed biofilm studies were cells labelled with 1) Primary antibody only, 2) Secondary antibody only, and 3) primary antibody conjugated to quantum dot secondary antibody.
4.2.8 Scanning Electron Microscopy (SEM)

4.2.8.1 Quantum Dots

One of the advantages of labelling cells with QDs is that they should also be visible as surface markers in the SEM, in a manner similar to surface imaging using immunogold labels. Both QD and gold markers can be recognised based on atomic number contrast in backscattered electron imaging mode in the SEM. Therefore, to determine the amount of label required to produce sufficient surface markers for observation by SEM, two different concentrations of secondary QD antibody, 40 µM and 100 µM were trialled. The conjugation was performed as described in section 4.2.7.2.

4.2.8.2 Immunogold

For direct comparison to these novel QD-labelled antibodies for *Cryptosporidium*, anti-rat secondary antibody conjugated with 12 nm colloidal gold, which has been used to label and image *Cryptosporidium* by SEM successfully before (Edwards et al., 2012) was purchased from Jackson ImmunoResearch. Prior to conjugation, this antibody was diluted (5% foetal calf serum in sterile 1 x PBS, pH 7.2) at 1:100 ratio. They were subsequently conjugated to the primary antibody by incubating the samples with this secondary antibody for 1 h at 37°C and rinsed several times with sterile 1 x PBS as described in section 4.2.7.2.
4.2.8.3 Poly-L-lysine / Cel-Tak Coverslip Coating

To allow for the examination of Cryptosporidium, 3 day-old biofilm-only and Cryptosporidium-exposed biofilm samples by SEM, the cells were immobilised and attached to coverslips using two different coverslip coatings, poly-L-lysine (Sigma) or Cel-Tak (Becton-Dickinson). Poly-L-lysine enhances electrostatic interaction between negatively-charged ions of the cell surface and positively-charged surface ions of attachment factors on the substratum surface. When adsorbed to the substratum surface, it increases the number of positively-charged sites available for cell binding. So far, it is only known that oocysts have negatively charged cell surfaces (Kuznar and Elimelech, 2004) and therefore it was unknown whether poly-L-lysine could also successfully adhere other Cryptosporidium stages. In contrast, Cel-Tak is a formulation of "polyphenolic proteins" extracted from a marine mussel with the capability to adhere to any type of cells. Therefore, these two adhesive solutions were trialled and compared.

Prior to coverslip coating, round coverslips (12 mm) were rinsed in 100% ethanol to remove any surface dirt and contamination and air dried in the laminar flow hood for 20 minutes. Coverslips were coated with poly-L-lysine for 20 minutes at room temperature. The coverslip was then washed with sterile water to remove excess poly-L-lysine and air dried in the laminar flow. The coated coverslip could be stored at 4°C for up to a week. To coat coverslips with Cel-Tak, 4 µL of Cel-Tak (stock solution) was spread evenly onto the coverslip and air dried in the laminar flow hood. When dry, the coverslip was washed with sterile distilled water and 100% ethanol. Cover slips were air dried again in a laminar hood. The prepared coverslips could be stored at 4°C for no more than 3 days.
Previously fixed experimental samples (biofilms/oocysts) were further fixed in 2.5% glutaraldehyde in sterile 1 x PBS solution and an aliquot of the sample (20 µL) was transferred to a prepared slide. To prevent sample dehydration at room temperature, the sample was left to stand in a humidified box for 30 minutes before being subsequently either 1) dehydrated through a series of ethanol concentrations and critical point dried, or 2) rapid frozen and freeze dried in preparation for SEM.

4.2.8.4 Critical Point Drying

Prior to the critical point drying process, dehydration steps were performed in a Biowave PELCO microwave. This was done with an increasing series of ethanol concentrations for 40 sec at 250 Watts, with no vacuum. Specifically, after allowing cell attachment to the coated coverslip, the coverslips were dehydrated with 50% ethanol, 70% ethanol, 95% ethanol, 100% ethanol and two times with fully dehydrated 100% ethanol. Samples in dehydrated 100% ethanol were then transferred into the boat that was specially designed for holding 12 mm circular coverslips for the critical point dryer. The boat containing samples was filled with 100% dehydrated ethanol as a substitution solvent and was immediately transferred into the critical point dryer. The chamber was flushed with the CO₂ liquid level always remaining above the level of the boat. The samples were then incubated for 15 minutes at room temperature until approximately 400 pounds per square inch (psi) was reached. Subsequently, the flushing steps were continually repeated until no more ethanol was detected in the chamber. After this, the samples were incubated for 30 minutes and flushed with liquid CO₂ one final time. The samples were then dried by slowly raising the temperature above 32°C and the pressure above 1200 psi.
Once the critical temperature and pressure were reached, the liquid transforms to gas and the drying process was complete. The chamber was vented slowly before removing the samples.

### 4.2.8.5 Freeze Drying

Towards the end of the PhD study, freeze drying became available and so was trialled in attempt to avoid frequent sample loss that occurred during the dehydration and critical point drying process. With this, the glutaraldehyde fixed sample attached to the coverslip was briefly rinsed in 150 mM ammonium acetate and blotted on filter paper to remove the excess liquid. The sample was then rapid frozen in liquid nitrogen slush and freeze dried using an Emitech K775X turbo pumped freeze drying system in a step wise fashion (-120°C to -65°C over 14 h; -65°C to +25°C over 10 h; hold at 25°C for 24 h).

### 4.2.8.6 Coating for Scanning Electron Microscopy

Samples for SEM need to be conductive, therefore, dried coverslips with attached oocysts (unexcysted/excysted) or 3 day-old *Cryptosporidium*-exposed biofilm samples were mounted on stubs with carbon tape. Depending on the purpose of the experiment, two different coatings were used: 1) Unlabelled samples were usually coated with 4 nm carbon and 5 nm platinum for stable, high resolution imaging using the in-lens secondary electron detector (SE), with an accelerating voltage of 3 kV. For these samples, the working distance can be kept very short (3-4 mm), hence high resolution information about the surface structure of specimens could be obtained.
using this detector. 2) To identify the surface markers of QDs and immunogold on Cryptosporidium parasites, samples were coated with 10 nm of carbon only, as a high atomic weight metal coating such as platinum will interfere with the identification of the QDs or gold labels when the specimen was observed using backscattered electron mode. As backscatter mode must be used to image the high atomic weight labels, the working distance between the detector and specimens was increased to 10 mm, and the accelerating voltage was increased to 10 kV. Under backscatter electron mode (BSE), the contrast generated between the heavy metal QD/immunogold labelling and the cells acts as an indicator of Cryptosporidium cells. Heavy metals always appeared much brighter than the unlabelled cell surface area, which is largely composed of H, N, O and C. In order to directly correlate the backscattered image of the heavy metal labels with cell structure, a secondary electron image was also acquired either separately or the signal was mixed (30% SE and 70% BSE). For this, a conventional secondary electron (SE2) detector was used instead of the in lens detector due to the longer working distance.

4.2.9 Correlative Confocal Microscopy with Scanning Electron Microscopy

4.2.9.1 SEM Grid Finder

Since 40 μM QD-labelled secondary antibodies were sufficient to observe Cryptosporidium vividly under the confocal microscope, this concentration was used throughout the study and an SEM finder grid was used to locate the same fluorescent cells from the confocal, in the SEM. The SEM grid finder pattern was made on a
sample mould (Figure 4.1). This pattern could be seen using both confocal and SEM, allowing for the establishment of directly correlative studies. The SEM finder grid pattern was transferred from the mould onto a 12 mm circular coverslip as described by Powell et al. (2009). Essentially, an SEM finder grid was placed flat on the 12 mm circular coverslip and sputter-coated with Pd/C for 1 minute (coverslips that will be poly-L-lysine coated) or 3 minutes (coverslips that will be Cel-Tak coated) to produce a clear outline of the SEM finder grid visible under both confocal microscopy and SEM.

Time was empirically determined according to the type of surface coating. Poly-L-lysine forms a thin film on the glass and therefore 1 minute of coating was sufficient to observe the grid using both inverted confocal microscopy and SEM (10 kV with BSE detector). For Cel-Tak, 3 minutes of coating was required to make a visible grid pattern due to its semi-transparent appearance on the coverslip. It was observed that the Pt/C thickness was sufficient to be observed at 10 kV with the SE2 detector. Hence, the BSE detector was not required and the in-lens detector was used. The in-lens detector provides better image resolution at 4 kV, but no grid pattern underneath the sample could be observed at this lower voltage. Therefore, 10 kV was also used for the in-lens detector in conjunction with using SE2 for simultaneous grid and sample observation.
After the pattern had been created, the coverslips were coated with poly-L-lysine or Cel-Tak as described above. Before transferring experimental samples to the prepared coverslip, all coverslip rims were sealed with a liquid blocker pen to prevent dehydration, immersion oil entry and contamination. This blocker remained on the slide permanently and hence was not removed for SEM imaging.

4.2.9.2 Correlative Method 1: Poly-L-lysine

Two different correlative methods were designed according to the type of coverslip coating. Unlike the Cel-Tak coated coverslip, poly-L-lysine formed a relatively thin film on the coverslip. As such, the method described below was more suitable for the poly-L-lysine coated coverslips (Figure 4.2). First, a thermanox coverslip was cut into a size of 22 x 22 mm and a circular hole was punched in the middle thermanox using a paper hole punch to support the coverslip during imaging. By doing this, the
working distance between the coverslip and inverted confocal microscope objective lens could also be decreased and better imaging resolution achieved. Thermanox was placed onto a square coverslip holder. The SEM grid finder coverslip was placed on top of the hole and 20 μL of experimental sample was transferred onto the coverslip and incubated in a humidified box for 20 minutes for cell attachment. After this, the sample was analysed by confocal microscopy. The cells of interest were imaged and their location on the coverslip was recorded for subsequent SEM observations. After confocal microscopy observation, the coverslip was gently lifted from the thermanox support and placed in 2.5% gluteraldehyde in sterile 1 x PBS at 4˚C. This sample could be stored for up to a week before further processing for SEM.

Figure 4.2: Correlative method 1
4.2.9.3 Correlative Method 2: Cel-Tak Coated

As Cel-Tak formed a thick film on the coverslip, the experimental sample could not be observed using correlative method 1, therefore the method described below was designed for all Cel-Tak coated coverslips (Figure 4.3). Experimental samples (20 µL) were placed onto the Cel-Tak coated coverslip and incubated in the humidified box for 15 minutes. During the incubation period, a clean sterile square coverslip (22 x 22 mm) was placed onto the square coverslip holder. After 15 minutes of incubation, the SEM coverslip was removed from the humidified box and placed onto the square coverslip. The coverslip was gently turned over so that the side with the experimental sample was facing down towards the square coverslip. After analysis by confocal microscopy and the location of the cells of interest had been recorded, the sample was fixed in 2.5% glutaraldehyde in sterile 1 x PBS. This sample could only be stored in the fridge for not more than one day and had to be immediately processed for SEM by freeze drying.
4.2.10 Transmission Electron Microscopy (TEM)

Transmission electron microscopy was performed to examine the stock solution of QD particles and check their sizes. A drop (6 µL) of the stock solution was placed on a C-filmed copper grid, air dried and imaged at 120 kV in a JEOL 2100 TEM. Images were collected on a Gatan ORIUS1000 digital camera.
4.3 Results

4.3.1 Live Imaging of Biofilm Structure (Optical Microscopy)

Figure 4.4 shows that 3 day-old *Salmonella* biofilms formed a relatively thin and transparent biofilm, hence, individual cells could be observed easily under the optical microscope. It was observed that the living biofilms consisted of a variable distribution of cells and cellular aggregates (Figure 4.4). Some bacteria were in the process of forming mature biofilms at the substratum (Figure 4.4). Movie 4.1 demonstrates that initial attachment was reversible as some bacteria were detached from the aggregated cells while some only attached briefly to the aggregated cells. In addition, some bacteria were fixed and rotating on the same spot, suggesting that flagella were involved in their attachment to the glass surface. Their attachment was further enhanced by staying close to other bacteria (Movie 4.1). This cell cluster was then transformed into irreversible attachment (second stage; Figure 4.4). The motility of cells within or in the centre of the cell cluster was decreased (darker patch; Movie 4.2). Only the surrounding cells were in the active form (lighter batch; Figure 4.4 and Movie 4.2). Cell clusters that became progressively layered were also observed and referred to as third stage at which the cell cluster may begin matrix production (Figure 4.4).
Figure 4.4: Live imaging of 3 day-old *Salmonella* biofilm. Square boxes indicate irreversible attachment. Circular boxes indicate biofilm maturation stage.
By day 5, *Salmonella* biofilms were matured and occupied most of the flow cell surfaces (Figure 4.5). Biofilms appeared as semi-transparent and opaque structures. It was observed that biofilm communities in this flow cell were formed by many microcolonies and appeared as multi-layered carpet-like biofilms. These layers were well separated by water channels. Although the internal content could not be viewed using conventional microscopy, based on the light refraction, the basal layer within the biofilm consisted of densely packed bacteria surrounded by a biofilm matrix (Figure 4.6). However, the detailed structure of densely packed bacteria could only be revealed by confocal microscopy.
Figure 4.5: Optical live imaging of 5 day-old *Salmonella* biofilms (side view). Bacteria formed carpet-like biofilms. Arrows indicate water channels.
4.3.2 Live Imaging of Biofilm Structure (Confocal Microscopy)

Due to the limitations of optical microscopy, live imaging of biofilm was performed using confocal microscopy. On day 1, side view (x-y view; Figure 4.8.A) showed that the bacteria were still in the process of forming biofilms as a large number of swimming bacteria were present and they appeared mostly as single cells, hence the average thickness was relatively thin (0.7 ± 0.4 µm; Figure 4.7), indicating that they were still an immature biofilm. As such, their biofilm structure was unlike that of 3 and 6 day-old biofilms, which had a more prominent architecture (Figure 4.8 B & C).
Figure 4.7: *Pseudomonas* biofilm thickness over a 6 day period. Graph showing biofilm thickness (mean ± standard error, n = 6) obtained from biofilm-only using the COMSTAT II quantitative programme. Significance was determined by a one-way ANOVA analysis (* P<0.05).

Biofilms thickness continued to increase over the next 3 day period (Figure 4.7). Although no significant increase in thickness was observed, many hay stack/mountain-like architectural microcolonies were formed (Figures 4.8.B and
4.9.B). This stage is referred to as ‘immature’, as the biofilm thickness were less than 100µm (21 ± 3 µm). This immature biofilm continued to grow and a significantly different biofilm thickness was observed between 1 and 6 day-old biofilm (105 ± 12 µm; P<0.05). This biofilm thickness fulfils the definition of a mature biofilm as previously defined by Davies et al.(1998), and hence was considered a mature biofilm. This also may explain why 6 day-old biofilms had the thickest basal layer (x-y view; Figure 4.8.C) and occupied most surfaces within the capillary (Figure 4.9.C).
Figure 4.8: Live imaging (confocal microscopy) – the side view (x-y view) of 1(A), 3 (B) and 6 (C) day-old biofilms. Scale bars = A & B: 50 µm; C: 100 µm.
Figure 4.9: Live imaging (confocal microscopy, top view) of 1 (A), 3 (B) and 6 (C) day-old Pseudomonas biofilms. Scale bars = 50 µm.


4.3.3 Live Imaging of *Cryptosporidium*-exposed Biofilms (Confocal Microscopy)

Prior to introducing *Cryptosporidium* into the biofilm system, the specificity of FilmTracer™ calcein red orange stain was tested in a mixed culture of bacteria and *Cryptosporidium*. Results showed that FilmTracer™ calcein red orange not only stained bacteria but also *Cryptosporidium* oocysts (Figure 4.10). This confirmed that FilmTracer™ calcein red orange stain is non-specific and stains any living cells. Therefore, *Cryptosporidium* specific antibodies were applied to avoid false readings.
Figure 4.10: *Cryptosporidium* oocysts that have been stained with FilmTracer™ calcein red orange. Elongated cells as seen in images A and B are bacterial cells. FilmTracer™ calcein red orange stained both oocyst walls and sporozoites. Arrow indicates sporozoite and, arrowheads indicate oocyst wall. B & C are images of the same cells. Image C is a brightfield image. Scale bars = 5 µm.

Oocysts pre-labelled with *Crypto-Cel* were introduced into the biofilm system for the ease of identifying and locating oocysts within the biofilm. Before doing so, the stability of the pre-labelled oocysts with *Crypto-Cel* oocyst-specific antibody in 10% TSB was tested for 1, 3, and 6 days. Figure 4.11 shows that oocysts did not lose
fluorescence over the 6 day period and that this stability was suitable for locating oocysts within the biofilm over the experimental period.

Furthermore, as FITC-Sporo-Glo™ antibody was to be introduced directly into living biofilms, the specificity of this antibody was also first determined in a mixed culture of bacteria and excysted oocysts. Figure 4.12.A shows that Sporo-Glo™ antibody is highly specific as it labelled Cryptosporidium only. In order to reduce the duration of the staining process, mixed cultures were incubated with FITC-Sporo-Glo™ antibody and FilmTracer™ calcein red orange simultaneously. All living cells were brightly stained (Figure 4.12.B) with FilmTracer™ calcein red orange. Unlike large oocysts, it was difficult to morphologically differentiate other smaller stages of Cryptosporidium from bacteria. However, when observing the same field of view at the wavelength for FITC-Sporo-Glo™ antibody (FITC fluorochrome), only Cryptosporidium was labelled and observed (Figure 4.12.C). Therefore, this observation confirmed that live biofilms could be post-stained with the combination of non-specific live cell stain and specific Cryptosporidium antibody at the same time.
Figure 4.11: *Cryptosporidium* oocysts pre-labelled with Crypto-Cel and incubated in 10% TSB for 1 (A & B), 3 (C & D) and 6 (E & F) days. Both confocal (A, C, E) and transmitted light images (B, D, F) were taken from the same cells. Scale bars = 5 µm.
After testing, these techniques were then applied to live, *Cryptosporidium*-exposed biofilm samples. One day-old biofilms showed some oocysts attached to the biofilm surface (Figure 4.13). On day 3, only surface oocysts were observed (Figures 4.14 and 4.15) and no FITC signal could be detected in the depths of the biofilms. On day 6, more oocysts were introduced yet no FITC signal was detected either from the
surface or within depths of the biofilms (result not shown). Thus, immunolabelling after chemical dispersion of biofilms was used instead of *in situ* staining.

Figure 4.13: Micrograph of side view (X-Y view) of composite images of oocysts (yellow) associated with *Pseudomonas* biofilms (red) in day 1. Scale bar = 10 µm.
Figure 4.14: Micrograph of side view (X-Y view) oocysts labelled with FITC-Crypto-Cel antibody (circled) associated with 3 day-old Pseudomonas biofilms. Scale bar = 20 µm.

Figure 4.15: Micrograph of side view (X-Y view) of magnified area from figure 4.14. FITC-Crypto-Cel labelled oocysts association with biofilms (circled) and attached to the surface. Scale bar = 10 µm.
4.3.4 FITC-Sporo-Glo™ Antibody Fluorescence Pattern

To study the fluorescence pattern of FITC-Sporo-Glo™ labelled antibody, unexcysted, excysted and other developmental stages of Cryptosporidium were used. No/weak fluorescence signal was detected from the unexcysted oocysts (Figure 4.16). However, when excysted oocysts were labelled with Sporo-Glo™ antibody, a series of changes in fluorescence intensity during the excystation process was observed (Figure 4.17). The fluorescence intensity of oocysts and sporozoites (Figure 4.17.C) increased once sporozoites had begun to exit into the external environment (Figure 4.17.D). The fluorescence intensity continually increased (Figure 4.17.E) as more sporozoites were released into the external environment (Figure 4.17.F) while at the same time, the fluorescence intensity of oocysts also started decreasing (Figure 4.17.G) and eventually only weak fluorescence intensity was detected when all 4 sporozoites had been released into the external environment (Figure 4.17.H).

In addition, cell-free culture was also performed to examine the fluorescence intensity of Sporo-Glo™ labelled Cryptosporidium at different stages. For the first 24 h, it was observed that sporozoites fluoresce intensely as compared to unexcysted oocysts (Figure 4.18.A & B) and the fluorescence intensity remained similar while they were in the process of transforming into trophozoites (Figure 4.18.C-F). Trophozoites pairing end to end that resembled the process of syzygy were also observed (Figure 4.18.E & F). After 48 h, the highly fluorescent trophozoite (2 x 2 µm, circular shape) appeared to fuse into an aggregate (Figure 4.19). The aggregation of trophozoites eventually led to the formation of type I/II meront after 72-96 h of incubation (Figures 4.20 and 4.21). Two morphological types of meront
were observed. One of them exhibited a grape-like aggregation of small, circular clumps (1 x 1 μm) fulfilling the description of type I meronts by Hijawi et al. (2004) (Figure 4.21). Type II meronts were confirmed by observing several clumps of oval shape cells (0.5 x 1.0 μm; Figure 4.21).
Figure 4.16: Confocal microscopy (A, C, E) and transmitted light images (B, D, F) of unexcysted oocysts labelled with FITC- Sporo-Glo™ antibody. Scale bars = 5 µm.
Figure 4.17: The changes in fluorescent signal of FITC- Sporo-Glo™ labelled excysted oocysts. Oocyst walls were weakly labelled while all sporozoites still remained inside the oocyst A & B). The fluorescent signal of the oocyst wall gradually decreased when two sporozoites were released from the oocyst, while the other two remained inside (C & D). The fluorescent signal of the oocyst continued to decrease as all the sporozoites were released into the external environment (E - H). Both confocal microscopy (A, C, E, G) and transmitted light images (B, D, F, H) were taken from the same cell. Scale bars: 2.5 µm.
Figure 4.18: The observation of Cryptosporidium stages from cell-free cultures using Sporo-Glo™ antibody. Both confocal (A, C, E) and transmitted light images (B, D, F) were taken from the same cells A & B show that sporozoites were brightly labelled as compared to the unexcysted oocysts (arrowhead). C & D show sporozoites (arrow) and trophozoites/merozoites (circled). E & F show trophozoites undergoing syzygy. Scale bars = A & B: 5 µm; C, D, E & F: 2 µm.
Figure 4.19: The observation of *Cryptosporidium* trophozoite stages from cell-free cultures using Sporo-Glo™ antibody. Both confocal (A, C, E) and transmitted light images (B, D, F). Images show aggregation of two (A & B), three (C & D) and ~twenty trophozoites (E & F). Scale bars = A-D: 2 µm; E & F: 5 µm.
Figure 4.20: The observation of *Cryptosporidium* type I meronts from cell-free cultures using Sporo-Glo™ antibody. Both confocal (A, C, E) and transmitted light images (B, D, F). Scale bars = 2 µm.
Figure 4.21: The observation of Cryptosporidium type II meronts from cell-free cultures using Sporo-Glo™ antibody. Both confocal (A, C, E) and transmitted light images (B, D, F). Scale bars = A & B: 2 µm; C & D: 1 µm; E & F: 2 µm.
4.3.5 Quantum Dots

4.3.5.1 Confocal Microscopy

Although all the developmental stages fluoresced brightly, photobleaching was observed for the Sporo-Glo™ labelled Cryptosporidium and good resolution images could not be consistently obtained within 10 minutes (data not shown). To resolve the photobleaching problem, QDs were selected to replace the FITC-Sporo-Glo™ antibody. QDs have a long absorption wavelength, therefore the optimum wavelength of QD-labelled excysted oocysts were examined. Results showed that the optimum wavelength for detecting Cryptosporidium was 488 nm, where all four sporozoites were well labelled as compared to 525 nm and 625 nm wavelengths (Figure 4.22). No photobleaching issues were encountered during the long experimental session.

A control check was performed for unexcysted and excysted oocysts, and 3 day-old biofilm exposed samples (Figures 4.23, 4.24 & 4.25). No fluorescent signal was detected from any samples labelled with only primary or only secondary antibodies. When samples were labelled with both primary and secondary antibodies, different fluorescent expressions were observed. Similar to FITC-Sporo-Glo™ labelling, unexcysted intact oocysts expressed weak fluorescence and no fluorescence was detected from sporozoites residing within the oocysts (Figure 4.23.E & F). The labelling pattern of QD-Sporo-Glo™ (Figures 4.24 E & F; 4.25) to excysted oocysts were also similar to that observed for FITC-Sporo-Glo™.

When biofilms were subjected to immunolabelling, only fluorescent expression of an excysted oocyst was detected from 3 day-old Cryptosporidium-exposed biofilms
A weak fluorescent signal was sometimes also detected from biofilm cell clusters (Figure 4.27). However, it was readily possible to differentiate developmental stages of *Cryptosporidium* from this non-specific binding based on fluorescence intensity (Figure 4.27).
Figure 4.22: Oocysts labelled with quantum dots-Sporo-Glo™ and observed at different wavelengths, 488 nm (A), 525 nm (B) and 655 nm (C). Bright field light imaging was also included (D). Scale bars = 5 µm.
Figure 4.23: Unexcysted oocyst labelled with: (A & B) Unconjugated Sporo-Glo™ (i.e. primary antibody only), quantum dot secondary antibody only (C & D), primary antibody conjugated to quantum dot secondary antibody (E & F). Images of confocal (A, C, E) and bright field light (B, D, F) were taken from the same cell. Scale bars = 2.5 µm.
Figure 4.24: Excysted oocyst labelled with: (A & B) Unconjugated Sporo-Glo™ (i.e. primary antibody only), quantum dot secondary antibody only (C & D), primary antibody conjugated to quantum dot secondary antibody (E & F). Images of confocal (A, C, E) and bright field light (B, D, F) were taken from the same cell. Scale bars = 2.5 µm.
Figure 4.25: A series of changes in fluorescence signal of quantum dot-Sporo-Glo™ labelled excysted oocysts. Excysted oocysts with two sporozoites residing within the oocyst were brightly expressed (A & B). However, once sporozoites were released from the oocyst, no fluorescent signal was detected from the oocyst wall (C & D). Both confocal microscopy (A & C) and bright field images (B & D) were taken from the same cell. Scale bars = 2.5 µm.
Figure 4.26: Three day-old Cryptosporidium exposed biofilms labelled with unconjugated Sporo-Glo™ (i.e. primary antibody only) (A & B), quantum dot secondary antibody only (C & D), primary antibody conjugated to quantum dot secondary antibody (E & F). Images G & H are magnified images of the oocyst in the square boxes in E & F. Images of confocal (A, C, E, G) and bright field (B, D, F, H) were taken from the same location. Scale bars = A & B: 20 µm; C & D: 10 µm; E & F: 50 µm; G & H: 2.5 µm.
4.3.5.2 Scanning Electron Microscopy

Due to limitations in confocal microscopy resolution, as in figure 4.27, it was still hard to verify the exact developmental stage of Cryptosporidium; hence, SEM was performed to provide high resolution imaging. Figure 4.28 shows that SEM not only provided the 3 dimensional structure of the cell but also revealed detailed morphological information about bacterial (Figures 4.28.A & B), 3 day-old biofilm matrix (Figures 4.28.C & D) and Cryptosporidium surface structure (Figures 4.28.E & F).
In addition, the contamination of yeast in biofilms could also be clearly identified by SEM (Figures 4.29 and 4.30). From the same sample, confocal microscope examination was performed but no fluorescence was detected (result not shown). This not only shows that Sporo-Glo™ antibody was highly specific but that budding yeast was not autofluorescent. Although SEM showed that the yeast have similar morphology, cell sizes and aggregation patterns to Cryptosporidium trophozoite stages (Figure 4.29), the presence of large amounts of yeast showed that they completely dominated biofilm samples (Figure 4.30).
Figure 4.28: Scanning electron micrographs of bacteria (A & B), 3 day-old biofilm matrix (C & D), excysted oocyst with emerging sporozoite (E) and aggregated oocysts (F). Scale bars = A: 2.5 µm; B: 5 µm; C: 2.5 µm; D: 10 µm; E: 1 µm; F: 2.5 µm.
Figure 4.29: The morphology of budding yeast identified within a 3 day-old Cryptosporidium-exposed biofilm system by scanning electron microscopy. Scale bars = A: 1 µm; B: 1 µm; C: 1 µm; D: 500 nm.
Figure 4.30: The identification of yeast contamination within a 3 day-old Cryptosporidium-exposed biofilm system by scanning electron microscopy. Images show that budding yeast dominated the biofilm system. Scale bars = A: 5 µm; B: 3 µm; C: 5 µm.

Initially, it was hypothesised that one of the advantages of labelling cells with QDs was that they could also be used as a identifying surface marker using SEM. Although extensive fluorescence intensity was expressed by Cryptosporidium under confocal microscopy, in the current study, the observation of low numbers and indistinctive QD surface markers by SEM (Figures 4.31 and 4.32) were not
sufficient to conclude that the observed stages were *Cryptosporidium*. It was suspected that this may be due to the low concentration of primary antibody. Consequently, a control check was performed with a similar concentration of primary antibody, but immunolabeled with gold-labelled secondary antibody, similar to Edwards *et al.* (2012). Results showed that unexcysted oocysts were weakly labelled (Figure 4.33.A & B). Unlike QD labelled oocysts, the immunogold markers were prominent on the surface of both excysted oocysts and sporozoites (Figure 4.33.C-F). To determine the conjugation efficiency of QD secondary antibody, 100 µM of secondary antibody was used. Figure 4.34 shows excysted oocysts labelled with QD markers of different shape and sizes.

![Figure 4.31: Excysted oocyst with sporozoite labelled with quantum dot-Sporo-Glo™ antibody. B is the magnified image of sporozoites using the back scatter detector. Only a low number of quantum dots were observed from the sporozoites. Scale bars = A: 1 µm; B: 200 nm. Arrows indicate probable quantum dots.](image-url)
Figure 4.32: Trophozoite (from 3 day-old Cryptosporidium-exposed biofilm) labelled with quantum dot- Sporo-Glo™ antibody. B is the magnified image of trophozoite using the back scatter detector. Only 2 quantum dot surface markers were observed from this biofilm sample. Scale bars = A: 1 µm; B: 200 nm. Arrows indicate probable quantum dots.
Figure 4.33: Immunogold conjugated with Sporo-Glo™ primary antibody was used to label unexcysted oocysts (A), excysted oocyst (C) and excysted oocyst with sporozoite (E). Oocyst surface (B), excysted oocyst surface (D) and sporozoite (F) were magnified and observed with back scatter electrons. Labelling of sporozoites (F) is extensive. Arrow indicates immunogold. Scale bars = A: 1 µm; B: 200 nm; C: 1 µm; D: 200 nm; E: 1 µm; F: 500 nm.
Figure 4.34: Excysted oocyst labelled with 100 µM quantum dots. The Sporo-Glo™ labelled oocyst was observed under scanning electron microscopy using secondary electrons (SE2) (A) and back scattered electrons (B). Arrows indicate probable quantum dots. Scale bars = 500 nm.

To further confirm these observations, the stock solution of the QD secondary antibody was also observed directly under SEM to determine whether the SEM settings were appropriate. Figure 4.35 not only showed that the current settings and the compositional contrast approach were appropriate but different shapes and sizes of QDs, which were irregular, were also observed.

Figure 4.35: Quantum dot particles (stock solution) observed by scanning electron microscopy using back scattered electrons. Quantum dots were bright as compared to the carbon background. Scale bar = 5 µm.
Transmission electron microscopy was then further used to determine the exact size and shape of the QDs. TEM results showed that QD’s shape was not consistent and that the sizes ranged from 4 nm to 20 nm (Figure 4.36). Even though 100 µM QD markers provided better observations, it was not a financially viable option. Therefore, an alternative approach to co-localising cells in the confocal and SEM was designed by using SEM grid finder patterning.

Figure 4.36: Transmission electron micrographs of quantum dot particles observed at different magnifications. Scale bars = A: 18 nm; B: 6 nm; C: 6 nm; D: 6 nm.
4.3.6 Poly-L-lysine / Cel-Tak Coverslip Coating

Two different solutions, poly-L-lysine and Cel-Tak were used to immobilise and attach cells onto the coverslip surface. Both of the surface coatings had advantages and disadvantages. Poly-L-lysine formed a thin layer and samples adhered to coverslips could be kept at 4°C for several days. Nevertheless, samples did not attach strongly and were regularly lost during critical point drying processing. Cel-Tak, which claimed to hold the samples better than poly-L-lysine, was trialled. However, the present study observed that Cel-Tak formed a thick film on the coverslip and could only attach the cells for a few hours before they began to detach again (Figure 4.37.A). After extended working hours (1 h), Cel-Tak lost its film structure and cells could not be located under SEM (Figure 4.37.B & C).
4.4 Discussion

4.4.1 Live Imaging of Cryptosporidium-exposed Biofilms

When pre-labelled Cryptosporidium oocysts were introduced into the biofilm system, several attempts to identify them within the depths of thick, mature biofilms failed. This observation is consistent with most studies, where only oocysts attached
to the biofilm surface were visualised and no oocysts were detected within the biofilm (Searcy et al., 2006; Wolyniak et al., 2009, 2010). However, this inability to visualise oocysts within the biofilm was probably due to two factors, i) imaging limitations of thick samples in a confocal microscope, and ii) the penetration, or lack thereof, of Sporo-Glo™ antibody into the biofilm. Firstly, confocal laser light was being attenuated by the thick biofilms, especially those above 50-100 µm thick (Pitts and Stewart, 2008). As such, single cell observation is only feasible at the biofilm surface or in areas where the biofilm is thin (<50 µm). It is hence impossible to observe Cryptosporidium within mature biofilms by confocal microscopy.

Secondly, the failure to observe Cryptosporidium developing stages with living biofilms may also be due to the Sporo-Glo™ antibody being unable to penetrate into the biofilms. A biofilm’s matrix can act as a barrier or molecular sieve to control the entrance of large molecules such as immunoglobulin G (Zhu et al., 2001). Although it is possible that more time may be required for antibody penetration, it can still be trapped within certain regions within the matrix (Thurnheer et al., 2003). Therefore to fluorescently label Cryptosporidium, the biofilms were dispersed and Cryptosporidium subsequently labelled and examined using confocal and SEM.

### 4.4.2 FITC-Sporo-Glo™ Antibody Binding Pattern

For ease of results interpretation, the binding pattern of Sporo-Glo™ antibody was studied in detail. Studies by Edwards et al. (2012) and Chapter 3 support the present observation that unexcysted oocysts often weakly expressed fluorescence, but once the oocyst had excysted, an increase in fluorescence intensity was observed. Furthermore, when all sporozoites were released from the oocyst, no fluorescent
signal or antibody binding was detected. This observation may be due to the fact that oocyst surface epitopes are altered once the oocyst has excysted and become inactive (Edwards et al., 2012). This is most likely to be influenced by the metabolic activity of internal sporozoites as it has been proposed that the oocyst surface proteins change according to metabolic inactivation (Kuznar and Elimelech, 2004; Edwards et al., 2012). On the other hand, it may be also indicated that Sporo-Glo™ will not label unexcysted oocysts, but once the suture opens, the antibody enters the oocyst and labels the sporozoites. The oocyst wall internally reflects this light giving the appearance of fluorescence, once the intensely fluorescing sporozoites start to leave the oocyst, the intensity of light reflecting within the oocyst wall diminishes and once the oocysts is fully excysted, light is no longer reflected and the oocysts wall appears dim.

Knowledge regarding the binding nature of this antibody would help to clarify some of these possible theories. Nevertheless, the characteristic binding of this antibody can be used as indicator of whether the excystation process was occurring within biofilms. It was also observed that other developmental stages - sporozoites, trophozoites, and type I & II meronts - also intensely expressed fluorescence. The morphologies of each developmental stage were similar to that observed in other cell-free studies (Hijjawi et al., 2004; Boxell et al., 2008; Hijjawi et al., 2010; Hammond et al., 2011).

However, photobleaching was the main problem identified with FITC-Sporo-Glo™ antibody. Identifying Cryptosporidium within biofilms or in the presence of massive numbers of bacteria was time consuming and the fluorescence intensity was often lost immediately when the fluorescently labelled parasite was excited by the laser light. Hence, instead of replacing the FITC-Sporo-Glo™ antibody with other
chemically fluorescent probes such as the Alexa series of fluorophores (Invitrogen), QDs were selected due to their very high photostability.

### 4.4.3 Quantum Dots (Confocal Microscopy)

Conventional fluorophores have a narrow excitation spectrum and require excitation by a specific wavelength (Jamieson et al., 2007; Resch-Genger et al., 2008). However, QDs have broad absorption spectra, allowing excitation by a wide range of wavelengths (Jamieson et al., 2007; Deerinck, 2008). Therefore, fluorescent signal of excysted oocysts at different wavelengths were tested and 488 nm was determined to be the optimum wavelength for this study. Based on the QD adsorption spectra (Figure 4.23), a shorter wavelength (~405 nm) may be able to provide better fluorescent signal of cells so as to reveal more cell structures, but this was limited by the microscope wavelength settings available on the instrument.

In addition, the findings of this study agree with those from other studies (Jaiswal and Simon, 2004; Deerinck, 2008) that QDs were much more sensitive to the confocal microscope laser light as compared to FITC-labelled Cryptosporidium. As in this study, the sensitteness was also not affected by the presence of interference from biofilm debris. Furthermore, the sensitivity of QDs was further enhanced by using QDs 655 that had a long emission wavelength so that more excitation light can be absorbed and provide a brighter signal. Therefore, this characteristic enables the QD-labelled Cryptosporidium to be easily distinguished from the clumps of dispersed biofilm sample and interference from autofluorescent particles.
Another characteristic of QDs that led to their selection in this study is their extreme photostability (Jaiswal et al., 2004; Jamieson et al., 2007; Li et al., 2011). Photobleaching was observed when FITC-labelled Cryptosporidium were subjected to laser excitation. This is a common problem when using organic fluorophores that usually undergo irreversible light-induced reactions upon optical excitation (Parak et al., 2005). This study reinforces the results of other studies that show that QDs are extremely stable and can undergo repeated cycles of excitation and fluoresce for hours with a high level of brightness and photobleaching threshold (Jaiswal et al., 2004; Parak et al., 2005; Jamieson et al., 2007; Tholouli et al., 2008; Li et al., 2011).

It has previously been reported QDs are almost 1000 times more stable than organic fluorophores (Tholouli et al., 2008). This is because the inorganic surface layers and the shielding of the core material can completely suppress the photo-oxidation over a long period of intense laser excitation imaging process (Resch-Genger et al., 2008). This property has made them suitable as biosensors and to detect Cryptosporidium in biofilm samples and to obtain high quality confocal images (Chapter 7).

In this study, it was observed that QD-labelled Cryptosporidium were stable for up to a week, after this, only weak fluorescent intensity was observed. This stability was relatively short as compared to other studies that usually can observe the samples for up to several months (Resch-Genger et al., 2008; Li et al., 2011). This may be due to the leaking of Cd\(^{2+}\) (Parak et al., 2005) from QDs after a prolonged binding to a different chemical interface (e.g., Cryptosporidium surface) (Dwarakanath et al., 2004).
4.4.4 Quantum dots (Scanning Electron Microscopy)

It was initially believed that QDs could be used as a surface marker for SEM analysis as the core shell of a QD is assembled by a tightly packed atomic lattice that can be directly detectable by both SEM and TEM (10-20 nm) (Jaiswal and Simon, 2004). Therefore, there was the potential use QDs to correlate and confirm the fluorescent signals from confocal microscopy with high resolution SEM observation. However, this study showed that QDs were not suitable for use as SEM surface marker for identifying Cryptosporidium within biofilms. Three main factors may have contributed to this observation. First, it was observed that QD particles are unlike nanogold, which has a consistent structure (circular in shape) and particle size (10 nm). TEM observation confirms that QDs have variable sizes (4-20 nm) and irregular shapes, which may contribute to the difficulty of identifying QDs on Cryptosporidium surfaces.

Secondly, from the confocal microscopy observations, it was suggested this antibody is not only a surface antibody but also a Cryptosporidium cytoplasm marker. This is because confocal microscopes, unlike conventional light microscopy, use laser light to optically section through the specimen allowing observation of internal structures. Hence if Sporo-Glo™ is a surface marker, the Cryptosporidium images generated from confocal microscopy should only show a rim of bright fluorescence around the outer edge of the cell, such as figure 4.17.G. However, sporozoites within oocysts (Figure 4.24), and some trophozoite (Figure 4.19) and type I/II meronts images (Figures 4.20 and 4.21) showed brightly labelled whole cells, hence, this indicates that this antibody has penetrated the cell and may also be effectively labelling
intracellular regions of *Cryptosporidium*. This would explain why few QDs were detected on the cell surface by SEM.

Although some of the larger sized QDs could be observed on the cell surface by SEM, this low number of QDs was not enough to confidently determine that the observed cells were labelled with the antibody, and therefore were *Cryptosporidium*. This is most likely due to the binding pattern of QDs, as 1 QD secondary antibody can usually bind up to several primary antibodies (Figure 4.38.A) (Lee *et al.*, 2004; Resch-Genger *et al.*, 2008). However, by using nanogold, 1 primary antibody can be bound by only 1 nanogold conjugated secondary antibody (Figure 4.38.B), and therefore many gold particles can be observed on the sporozoite surface (Figure 4.33.F).

![Figure 4.38](image.png)

**Figure 4.38:** The binding pattern of quantum dot (A) and nanogold secondary antibody (B) to Sporo-Glo™ primary antibody. Image adapted and modified from Lee *et al.* (2004).

As such, 100 µM of QDs were used to saturate primary antibody binding sites and indeed, more QDs were observed on the surface, but their variable shape and brightness are not as distinctive as gold particles. As such, this study concludes that
QD-conjugated antibody is an excellent tool for fluorescent imaging but is not suitable for correlative SEM observation. This study showed that the immunogold approach may still be the best way to label surface epitopes for SEM analysis (Edwards et al., 2012).

4.4.5 Correlative Confocal and Scanning Electron Microscopy Study

The SEM finder grid was instead used to correlate confocal microscopy observations with SEM. Two dehydration methods were used according to the type of surface attachment coating used: poly-L-lysine or Cel-Tak. The current study observed that many cells were lost during the dehydration and critical drying processing steps, with cells that were observed by confocal no longer present on the coverslip when imaged with SEM. This is most likely due to poly-L-lysine being unable to adhere the cells firmly. Samples missing after critical point drying has been previously reported (Inoue and Osatake, 1988) and it is suspected to occur when liquid carbon dioxide runs through the samples. As such when the freeze drying option became available, Cel-Tak was used instead to adhere the cells onto the coverslip. Although the adherence strength of Cel-Tak was often lost after a few hours of observation, Cel-Tak appeared to hold cells better than poly-L-lysine as several distinctive stages were observed and correlated well with confocal data (Chapter 8).

Even though the freeze drying process required a longer processing time, the cells do not need to go through a series of liquid ethanol dehydration and drying stages thereby reducing the chances of losing cell samples during the washing and drying
process. In addition, when observations from critical point drying and freeze drying were compared, fewer artefacts such as salt crystals were observed on the freeze drying cells. Moreover, like other studies (Billings-Gagliardi et al., 1978; Inoue and Osatake, 1988), it appears that freeze drying retained the dimensions of Cryptosporidium developmental stages better than ethanol dehydration and critical point drying. As such, the images and data obtained by the freeze drying method were considered to be of better quality than those obtained from conventionally dehydrated and critical point dried samples.

4.4.6 Conclusion

In conclusion, this study has successfully optimised immunolabelling, confocal and SEM microscopy protocols for studying the extracellular stages of Cryptosporidium in the dispersed biofilms. In addition, by labelling Cryptosporidium cells with Sporo-Glo™ antibody, Cryptosporidium developmental stages within biofilms were not only easily identified but could also be used as an indicator for the excystation process. As such, the combination of these techniques will allow for a morphologically definitive conclusion to be drawn about the fate of Cryptosporidium oocysts after exposure to aquatic biofilms.

4.5 References


5

Multiplication of the Waterborne Pathogen *Cryptosporidium Parvum* in Aquatic Biofilm Systems
5.1 Introduction

Biofilms are highly efficient and stable ecosystems (Declerck et al., 2009) that are formed mainly by bacteria (Fisher et al., 2000; Dunne, 2002; Wingender and Flemming, 2011), creating a favourable micro-environment that can support the survival and growth of other micro-organisms under prolonged periods of environmental stress (Declerck et al., 2009). Biofilms have been shown to serve as an environmental reservoir for Cryptosporidium oocysts in aquatic environments (Searcy et al., 2006; Angles et al., 2007; Helmi et al., 2008) and may be responsible for the occurrence of sporadic Cryptosporidium outbreaks (Howe et al., 2002). An increasing number of in-vitro and cell-free cultures studies (Hijjawi et al., 2002; Hijjawi et al., 2004; Karanis et al., 2008; Zhang et al., 2009; Hijjawi et al., 2010), have demonstrated that Cryptosporidium may not be an obligate intracellular parasite and can in fact multiply extracellularly. Therefore, there is a current need to better understand Cryptosporidium behaviour in biofilm environments, especially in relation to water distribution systems (Fisher et al., 2000; Angles et al., 2007), and to investigate whether Cryptosporidium oocysts captured within biofilms can utilise this nutrient rich micro-environment to survive and multiply.

Hence, the aim of this study was to investigate whether biofilms can support the multiplication of Cryptosporidium in aquatic environments. Model flow cell biofilm systems were developed and confocal laser scanning microscopy coupled with image analysis was used to quantitatively compare biofilm thickness between pure biofilm cultures and biofilms exposed to Cryptosporidium oocysts. Quantitative PCR was used to determine changes in the number of Cryptosporidium within the biofilm system over a 6 day period.
5.2 Materials and Methods

5.2.1 Bacterial Strains and Media

Wild type *Pseudomonas aeruginosa* bacteria (PA01) were prepared and used to establish the biofilms as previously described in Chapter 2 (Section 2.2.1).

5.2.2 Cryptosporidium Purification

*Cryptosporidium parvum* cattle genotype (Swiss cattle C26) oocysts were purified and prepared as previously described in Chapter 2 (Section 2.2.2).

5.2.3 Flow Cell Biofilm Systems

Flow cell biofilm systems were set up as previously described in Chapter 2 (Section 2.2.3). Biofilm-free and biofilm-only controls were also set up simultaneously (Section 2.2.3). All experiments were repeated three times.

5.2.4 Biofilm Thickness

Film Tracer calcein red orange (Invitrogen) was used to stain the biofilm for confocal laser scanning microscopy imaging and analysis as previously described in Chapter 4 (Section 4.2.6.1). The z-stack images of *Cryptosporidium*-exposed
biofilms were then analysed with COMSTAT II (Heydorn et al., 2000) and compared to the thickness of biofilm-only control as previously described in Chapter 4 (Figure 4.7).

5.2.5 DNA Extraction

Following thickness measurements, biofilms were dispersed by incubation in 500 nM sodium nitriporusside and the cell suspensions were collected and prepared as previously described in Chapter 2 (Section 2.2.4).

Aliquots of flow cell biofilms (100 µL) and effluent biofilms (500 µL) were used for DNA extraction. The full description of DNA extraction is provided in Chapter 2 (Section 2.2.5). The purified DNA was then eluted in a final volume of 50 µL in the DNA suspension buffer provided with the kit.

5.2.6 Quantitative Polymerase Chain Reaction (qPCR)

Standard curves were constructed using five genomic DNA triplicates extracted from a known number of oocysts and serially diluted at 1:10 dilution ratio, calibrated to correspond from $10^0$ to $10^5$ oocysts. *C. parvum* specific primers used in this study were glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers. Details of the qPCR assay are described in Chapter 2 (Section 2.2.6.1).

As previously described in Chapter 2, the dilution factors for detecting *Cryptosporidium* were 1:100 in 1 and 3 day-old *Cryptosporidium*-exposed biofilms and 1:1000 for 6 day-old *Cryptosporidium*-exposed biofilms. Biofilm-free and 6
day-old biofilm-only samples (negative controls) were diluted according to the respective dilution factor of the biofilm waste samples. The total number of Cryptosporidium present in each experiment was obtained by adding the number of parasites within the biofilm and the number of parasites within the effluent from each experiment. Biofilm-free controls were similarly analysed to provide a comparable baseline of the number of Cryptosporidium parasites within the system, independent of the biofilm.

5.2.7 Statistical Analyses

All experimental data are presented as mean ± the standard error of the mean. Analyses were performed using GraphPad Prism, version 5 (GraphPad Software, San Diego, CA). One-way ANOVA analysis of variance was performed to determine whether there were significant differences in (i) thickness between 1, 3 and 6 day-old Cryptosporidium-exposed biofilms, (ii) amount of Cryptosporidium detected from 1, 3 and 6 day-old Cryptosporidium-exposed biofilms (flow cell). Two-way ANOVA analysis of variance was used to determine whether there were significant differences in (i) thickness between Cryptosporidium-exposed biofilms and biofilm-only controls, (ii) the total number of Cryptosporidium detected at the completion of experimental and biofilm-free experiments.
5.3 Results

5.3.1 Comparison of Biofilm Thickness: Biofilm-only vs. Cryptosporidium-exposed Biofilm

When Cryptosporidium oocysts were introduced into the biofilm system, 1 day-old Cryptosporidium-exposed biofilms formed immature biofilms that were 30.1 ± 2.8 µm thick (Figure 5.1). Unlike biofilm-only experiments, a significant (P<0.05) increase in biofilm thickness was observed in Cryptosporidium-exposed biofilms after only 3 days (120.8 ± 3.6 µm; Figure 5.1). The thickness of these 3 day-old Cryptosporidium-exposed biofilms (~100 µm) could be classified as the penultimate stage of biofilm development (Davies et al., 1998; Sauer et al., 2002) and was considered to be a mature biofilm. No significant (P>0.05) increase in the thickness of this mature biofilm was observed from day 3 to day 6 in Cryptosporidium-exposed biofilms, with 6 day-old biofilms found to be 136.0 ± 6.8 µm thick (Figure 5.1).

Overall, all Cryptosporidium-exposed biofilms were thicker than biofilm-only controls, but the level of significance varied. Although both biofilm-only controls and Cryptosporidium-exposed biofilms formed immature biofilms on day 1 and mature biofilms by day 6, Cryptosporidium-exposed biofilms were always significantly (P<0.01) thicker than the biofilm-only controls. In addition, by day 3, Cryptosporidium-exposed biofilms had formed mature biofilms and thus had matured significantly (P<0.0001) faster than the comparable 3 day-old immature biofilms in the biofilm-only control.
5.3.2 Association of *Cryptosporidium* with Biofilms

Overall, qPCR revealed that the number of *Cryptosporidium* parasites retained within the biofilm increased significantly (P<0.05; Figure 5.2) and continually over time, as the biofilm increased in thickness and matured. When 1 and 3 day-old *Cryptosporidium*-exposed biofilms were compared, a significant (P<0.05) increase in both parasite number (Figure 5.2) and biofilm thickness (Figure 5.1) was observed.
over time. However, even when the Cryptosporidium-exposed biofilm had reached maturity and no longer underwent any increase in thickness (day 3; Figure 5.1), a significant (P<0.05) increase in Cryptosporidium numbers continued to be observed until day 6 (Figure 5.2).

![Figure 5.2: Quantitative analysis of Cryptosporidium number within 1, 3 and 6 day-old Cryptosporidium-exposed biofilms. The number (mean ± standard error) of Cryptosporidium retained within Pseudomonas biofilms after 1, 3, and 6 days of exposure (n = 6 for each time period), as determined using a DNA-based qPCR approach. Significance was determined by a one-way ANOVA analysis (* P<0.05).](image)

Similarly, for the total number of parasites in the flow system (i.e. both those retained within the biofilm + those recovered from the effluent), an increase in the total number of Cryptosporidium was observed in both 1 and 3 day-old Cryptosporidium-exposed biofilms, but these were not significantly (P>0.05)
different to biofilm-free controls (Figure 5.3). However, after 6 days, Cryptosporidium-exposed biofilms contained 2-3 fold more Cryptosporidium than that of the biofilm-free controls, which reflected a highly significant (P<0.001) increase in parasite numbers when a biofilm was present (Figure 5.3). This significant increase in Cryptosporidium numbers within the biofilm flow system over the 6 day period implies that Cryptosporidium was not simply captured and accumulated but underwent multiplication within the biofilm system.

**Figure 5.3:** Comparison of the total number of Cryptosporidium present in Cryptosporidium-exposed biofilms and biofilm-free control systems. Graph showing the total number (mean±standard error) of Cryptosporidium (retained in biofilm + effluent) determined at the end of each experiment (1, 3 and 6 day) for Cryptosporidium-exposed biofilms (black bar) (n = 3) and Cryptosporidium only (biofilm-free) control systems (white bar) (n = 3), as determined using a DNA-based qPCR approach. Significance was determined by a two-way ANOVA analysis with a post hoc test of significance (* P<0.001).
5.4 Discussion

This is the first study to demonstrate a significant increase in Cryptosporidium numbers over time within a biofilm system, highlighting that biofilms can readily provide a suitable environment for not only the retention, but also the multiplication of Cryptosporidium parasites, in aquatic environments. Wolyniak et al. (2009) have shown that the number of oocysts retained within biofilms remained constant while oocysts were continually supplied to the biofilm system. However, the apparent discrepancy between these results and those presented here can be explained by differences in the type of biofilm and methods used to detect Cryptosporidium. In Wolyniak et al. (2010), natural biofilms were used with filter-sterilised creek water used as the medium. Therefore, when compared to artificial Pseudomonas biofilms produced in this study, their biofilms would have a different community structure and nutrient levels. In addition, through the use of qPCR techniques, presence analyses quantified not only the oocysts within the system but also other Cryptosporidium life stages that were produced through multiplication.

Cell-free culture studies by Hijjawi et al. (2004) and Zhang et al. (2009) support the observation that Cryptosporidium can multiply extracellularly, and that encapsulation within a host cell is not essential for multiplication to occur. This ability to multiply either intracellularly or extracellularly suggests that Cryptosporidium i) is capable of extracting the nutrients required for growth and multiplication from the surrounding environment, ii) is not an obligate intracellular parasite, and iii) may be physiologically as well as genetically similar to the closely-related gregarines. Further imaging (Boxell et al., 2008; Valigurová et al., 2008; Hijjawi et al., 2010; Edwards et al., 2012) and flow cytometry-based studies (Valdez
et al., 1997; Vesey et al., 1997; King et al., 2009) are needed to fully characterise the additional life stages that may be produced within the biofilm. This could be achieved using specific life stage antibodies such as the commercial Sporo-glo from Waterborne Inc. (Boxell et al., 2008; Hijjawi et al., 2010; Edwards et al., 2012) and through the use of high resolution electron microscopy (Valigurová et al., 2008; Borowski et al., 2010; Edwards et al., 2012).

The presence of *Cryptosporidium* was also shown to significantly affect biofilm development and maturation. *Cryptosporidium*-exposed biofilms were found to form mature biofilms significantly faster than biofilms forming without exposure to *Cryptosporidium*. Consistent with this, Singleton et al. (1997) also showed that biofilms that contain both prokaryotic and eukaryotic cells often formed extensive dense and thick mature biofilms. Although it is not possible to determine what proportion of the increase in the mature biofilm thickness was due to increases in the number of bacteria or of *Cryptosporidium*, qPCR analyses in this study demonstrated that even very immature biofilms were capable of capturing and accumulating *Cryptosporidium* oocysts. These may be incidentally incorporated into cell clusters during the biofilm aggregation process. Additionally, during transformation from an immature to a mature biofilm, matrix and water channels that form on the biofilm surface may have enhanced the adhesion of oocysts and also encased both parasites and bacteria, trapping those that were already retained within the biofilm (Rickard et al., 2003; Wolyniak et al., 2009). However, while the thickness and maturation rate of the biofilm was affected by *Cryptosporidium*, the two factors were not strongly correlated, thus biofilm thickness cannot be reliably used as an indicator of the number of *Cryptosporidium* residing within the biofilm, a finding also concluded by other studies (Okabe et al., 1998; Wolyniak et al., 2009, 2010; Okabe et al., 2011).
In conclusion, this study shows that biofilms not only serve as an environmental reservoir for oocysts, but are also capable of supporting *Cryptosporidium* multiplication in an aquatic environment. The presence of biofilm on the pipes of water systems may pose a public health threat as *Cryptosporidium* residing within biofilms may increase in quantity over time, before being released into the water supply. With this, authorities should take into consideration the ability of *Cryptosporidium* to multiply within biofilms in aquatic environments when designing preventive measures to control *Cryptosporidium* contamination in water distribution systems.

5.5 References


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Flow Cytometry Analysis: Revealing the Fate of Cryptosporidium Oocysts in Aquatic Biofilms
6.1 Introduction

Until now, studies performed on biofilms and Cryptosporidium have mainly focussed on Cryptosporidium oocyst attachment onto biofilms (Wolyniak et al., 2009, 2010), but the fate of oocysts after attachment has never been examined. Biofilms are highly organised community that have stable and synergistic microconsortia internally (Wingender and Flemming, 2011) and hence it is capable of harbouring a wide range of microorganisms (Percival and Walker, 1999) in aquatic environments. Initial qPCR data (Chapter 5; Figure 5.3) show that Cryptosporidium can multiply within biofilms, with a 2-3 fold increase in Cryptosporidium number observed over a 6 day period.

For multiplication to occur, oocysts must first undergo an excystation process (Neumann et al., 2000). The excystation process of Cryptosporidium involves a complex biochemical signalling mechanism that is stimulated by chemical and physical factors from the surrounding environment (Neumann et al., 2000; Kato et al., 2001; Hijjawi, 2010; Karanis and Aldeyarbi, 2011). This process creates a cleft-like opening along a suture at one pole of the oocyst to release sporozoites into the external environment (Reduker et al., 1985). As such, excysted oocysts have a distinctively different morphology from intact oocysts and this can be detected by flow cytometry (Vesey et al., 1997; Neumann et al., 2000; Kato and Bowman, 2002; King et al., 2009).

The aim of this study was to use flow cytometry to track the fate of oocysts after exposing them to biofilms. Effluent sample collected from each experiment day was analysed by flow cytometry in this study. Oocyst populations in the effluent samples were fluorescently labelled with Cy5-labelled Cryptosporidium oocyst-specific
antibody (Crypt-a-glo™, Waterborne Inc). Using forward scatter analysis for measuring cell size, morphological changes in oocysts when they encounter biofilms were monitored over a 6-day period. The flow cytometry results from effluent samples were compared with cell-free culture observations using both flow cytometry and confocal microscopy analysis. A biofilm-free system was also used to determine whether Cryptosporidium can excyst in the absence of aquatic biofilms.

6.2 Materials and Methods

6.2.1 Bacteria Preparation

Wild type Pseudomonas aeruginosa (PA01) was used for the biofilm and were maintained as previously described in Chapter 2 (Section 2.2.1).

6.2.2 Cryptosporidium parvum Oocysts Preparation

Cryptosporidium parvum oocysts were prepared as described in Chapter 2 (Section 2.2.2).

6.2.3 Biofilm System

Cryptosporidium-exposed biofilm capillary biofilm systems were set up as detailed in Chapter 2 (Section 2.2.3). Biofilm-free controls were also set up as previously described in Chapter 2 (Section 2.2.3). All experiments were repeated 3 times.
6.2.4 Biofilm Dispersion

Both *Cryptosporidium*-exposed and biofilm-free systems were dispersed with 500 nM of sodium nitroprusside and the suspension was collected as previously described in Chapter 2 (Section 2.2.4).

6.2.5 Cell-free Culture

Cell-free culture was performed to determine morphological changes in oocysts during the excystation process. *Cryptosporidium* oocysts (4 x 10^6) were excysted as previously described in Chapter 3 (Section 3.2.4). Approximately 10^6 oocysts were immediately post-fixed with 2.5% paraformaldehyde in sterile 1 x PBS for 20 minutes at 4°C. The cells were pelleted and resuspended to a final volume of 200 µL. This sample was stored at 4°C for further flow cytometry analysis and is hereafter referred to as “excysted” oocysts. The remaining excysted oocysts were kept in a maintenance media for a further 60 minutes or 24 h at 37°C. These samples are referred to as “60 minutes” and “24 h” culture samples respectively.

6.2.6 Immunolabelling

The oocyst-specific antibody, Cy5-Crypt-a-glo™ was used in this study. The specificity and optimisation for this antibody were previously tested (Chapter 3; Figures 3.3 and 3.4). All samples (200 µL) including unexcysted *Cryptosporidium* oocysts (1 x 10^6), *Cryptosporidium*-exposed biofilms, biofilm-free controls and
Cryptosporidium cell-free cultures were immunolabeled with Cryptosporidium oocysts specific antibody (5.0 µg/mL; Crypt-a-glo™; Waterborne Inc) according to the method described in Chapter 3 (Section 3.2.5.1). Except for cell-free culture samples, all other samples were resuspended to a final volume of 200 µL.

Cell-free culture samples were resuspended in 400 µL of sterile 1 x PBS and an aliquot of each cell-free culture sample (200 µL: 5 x 10⁵ oocysts) was taken and processed for morphological analysis by confocal microscopy. The rest of the samples were resuspended in a final volume of 200 µL with sterile 1 x PBS solution and analysed by flow cytometry on the same day.

### 6.2.7 Confocal Microscopy Analysis

All confocal pictures were taken on a Leica TSC-SP2 AOBS microscope using a laser excitation source of 649/688 nm (Cy5 wavelength).

### 6.2.8 Flow Cytometry

All prepared samples were analysed using flow cytometry (FACSCalibur, Becton Dickinson, Australia). The purpose of each experiment is summarised in Table 6.1.

Settings for flow analysis of Cy5-labelled oocysts as described in Chapter 3 (Section 3.2.5.5) were used here. Cy5-Crypt-a-glo™-labelled unexcysted oocysts stock suspensions were used to define an area in the flow cytometry profile where Cryptosporidium oocysts were most likely to be found. Two different populations
were similarly used to differentiate gated areas for each of the physiological states of oocysts, “intact” (4-5 µm), and “excysted” (< 4 µm). The information of these two populations were previously been discussed in Chapter 3. The collected data were analysed using the FlowJo software (TreeStar).
Table 6.1: Summary of the purpose of each experiment performed in this study.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Biofilm</th>
<th><em>Cryptosporidium</em> Oocysts</th>
<th><em>In-vitro</em> Excystation</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stock oocysts</td>
<td>-</td>
<td>+</td>
<td>No</td>
<td>Determine gated area of intact oocysts</td>
</tr>
<tr>
<td>Cell-free culture (unexcysted, excysted, 60 minutes &amp; 24 hours)</td>
<td>-</td>
<td>+</td>
<td>Yes</td>
<td>Monitor morphology changes following excystation and determine gated areas for these</td>
</tr>
<tr>
<td><em>Cryptosporidium</em>-exposed biofilm (1, 3 &amp; 6 days)</td>
<td>+</td>
<td>+</td>
<td>No</td>
<td>Monitor oocyst populations in biofilm over time</td>
</tr>
<tr>
<td>Biofilm-free (1, 3 &amp; 6 days)</td>
<td>-</td>
<td>+</td>
<td>No</td>
<td>Determine if excystation occurs without biofilm</td>
</tr>
</tbody>
</table>
6.3 Results

6.3.1 Observation of Morphological Changes to Oocysts in Cell-free Culture

Flow cytometry and confocal microscopy were used to observe the morphological changes of oocysts and to correlate this with the observed oocyst populations of Cryptosporidium-exposed biofilms samples. Flow cytometry showed that all unexcysted oocysts were larger than 4 µm before excystation with acid water, hence this population was designated as “intact oocyst” population (Figure 6.1.A). A small population of oocysts < 4 µm were identified immediately following the excystation procedure (Figure 6.1.B). After further incubation in maintenance media for 60 minutes or 24 h, a wide range of oocyst sizes ranging from 1 to 4 µm was observed in the excysted oocyst population (Figure 6.1.C & D). Confocal and bright field microscopy revealed that this population was formed by excysted oocysts exhibiting variable sizes, which coincided well with the flow cytometry profile (Figure 6.2).
Figure 6.1: Flow cytometric profiles of oocyst populations from the cell-free culture study. Four different samples were analysed, unexcysted oocysts sample (A), excysted oocysts sample (B), 60 minutes (C) and 24 h (D). All samples were stained with Cy5-labelled Crypt-a-glo™ monoclonal antibody. Unexcysted *C. parvum* oocysts (A) were used to define an area where intact oocysts would most likely to be found in cell-free culture samples.
Figure 6.2: Superimposed images of confocal and bright field microscopy of excysted oocyst populations from 60 minutes samples. All samples were stained with Cy5-Crypt-a-glo™ monoclonal antibody. A represents well intact oocysts. Note that the bulky appearance of oocyst indicates the presence of its internal components such as sporozoites. B shows the internal contents of oocysts excysting into the surrounding environment. C represents an empty oocyst. D shows an excysting oocyst that may have a sporozoite still remaining inside. E and F show empty oocysts undergoing a series morphological changes and size reduction. Scale bars = 5 µm.
6.3.2 Association of *Cryptosporidium* with Biofilms

After the exposure of unexcysted oocysts to *Pseudomonas* biofilms, several distinctive fluorescently labelled populations were observed in the effluent samples. This included “intact” oocyst populations, which indicates, not surprisingly, that not all oocysts were accumulated within the immature and mature biofilms (Figure 6.3). For the oocysts that had been trapped and released back into the flow, it is evident that some of them had undergone excystation within the immature biofilms from day 1 (Figure 6.3.B). This population was similar to the “excysted” oocyst population seen in the cell-free culture. As the biofilms matured from day 3 to day 6, it also appeared that more oocysts underwent excystation (Figure 6.3.C-D).

Additionally, after 3 days a prominent “biofilm component” population was also identified. This “biofilm component” population was similar to the population found in the biofilm-only controls (Chapter 3; Figure 3.12). Thus, this population was assigned to be part of the biofilm component. Interestingly, an unknown population that could only be observed in 6 day-old *Cryptosporidium*-exposed biofilms was identified (Figure 6.3.D). This population expressed lower fluorescence intensity but had similar cell sizes to that of intact oocysts populations (4-5 µm). This population was postulated to be the non-viable oocyst population.

When similar batches of unexcysted oocysts were introduced into the biofilm-free system, only “intact” oocyst populations were identified during the study (Figure 6.4). Neither “excysted” nor other fluorescent populations such as “biofilm component” or “non-viable” populations were found from the samples. These observations confirmed that oocysts did not excyst in the absence of a biofilm.
Figure 6.3: Flow cytometric profiles of oocyst populations from 1, 3 and 6 day-old *Cryptosporidium*-exposed biofilm samples. All samples were labelled with Cy5-Crypt-a-glo™ monoclonal antibody. Unexcysted *C. parvum* oocysts stock (A) was used to define an area where intact oocysts would most likely to be found 1 (B), 3 (C) and 6 (D) day-old *Cryptosporidium*-exposed biofilm samples (effluent).
Figure 6.4: Flow cytometric profiles of oocyst populations from 1, 3 and 6 day-old biofilm-free samples. All samples were labelled with Cy5-Crypt-a-glo™ monoclonal antibody. Purified *C. parvum* oocyst stock suspensions (A) was used to define an area where intact oocysts would most likely be found from 1 (B), 3 (C) and 6 (D) day-old biofilm free samples.
6.4 Discussion

This study used flow cytometry to track the fate of oocysts after exposure to biofilm. It is confirmed that oocysts can excyst within aquatic biofilms, based upon evidence of variable-sized oocyst populations. Several flow cytometry studies support this observation, validating that intact oocysts can be easily differentiated from excysted oocyst populations by flow cytometry (Vesey et al., 1997; Neumann et al., 2000; King et al., 2009). In addition, parallel confocal microscopy observations of cell-free culture experiments confirmed that the excysted oocyst populations were formed by excysted oocysts of variable sizes, but not of degraded oocysts. Furthermore, the absence of “excysted” oocyst populations in the biofilm-free samples demonstrated that Cryptosporidium did not excyst without the presence of a biofilm in an aquatic environment.

Similar to Wolyniak et al. (2010), oocyst populations were observed throughout the experiment, but these data further demonstrate that these oocysts were actually present as two different oocyst populations - excysted and intact oocysts. This excystation process is mostly due to the exposure of oocysts to bacterial proteases in the biofilms. Nasser et al. (2007) showed that exposure to Pseudomonas aeruginosa protease resulted in digestion of the oocysts’ cell wall. This suggests that they may be constantly exposed to bacterial proteases, causing the release of sporozoites and leading to subsequent multiplication within the biofilms.

In addition, biofilms may also have the capability to influence the number of Cryptosporidium residing within biofilms. Although further investigations are required to determine the exact identity of the “non-viable” oocyst populations from the 6 day-old Cryptosporidium-exposed biofilms, this study postulates that biofilms
have a saturation point to control the amount of Cryptosporidium residing within biofilms. This is because Cryptosporidium also rely on and compete for the nutrients within biofilms to survive; and hence may eventually threaten the survival of bacterial populations within biofilms. Furthermore, this population was not seen in biofilm-free controls. Therefore, it is speculated that biofilm may have inactivated these captured oocysts and halted their excystation process.

In conclusion, this study has successfully shown that Cryptosporidium can excyst in aquatic biofilm systems, and they may play an important role in shaping Cryptosporidium life cycle in aquatic environments. Undoubtedly, the interaction between Cryptosporidium and biofilms warrants further investigation.

### 6.5 References


7
Confocal Microscopic Analysis of Cryptosporidium Interaction within Biofilms
7.1 Introduction

*Cryptosporidium* has a complex life cycle (Valigurová et al., 2008; Borowski et al., 2010) and it can be difficult to identify and discriminate between each life cycle stage using conventional optical microscopy. In addition, certain stages of *Cryptosporidium* such as trophozoites and merozoites are very small and may be mistaken as yeast and fungus contamination (Woods and Upton, 2007). Thus, *Cryptosporidium*-specific fluorescent antibodies are an important tool in identifying and confirming extracellular stages of *Cryptosporidium* (Boxell et al., 2008). Several studies have utilised this superior method and have shown that *Cryptosporidium* can multiply extracellularly (Boxell et al., 2008; Edwards et al., 2012). Currently, there is only one commercial antibody on the market, Sporo-Glo™ antibody (Waterborne Inc.), which specifically targets the developmental stages of *Cryptosporidium* (Boxell et al., 2008). The binding pattern of Sporo-Glo™ antibody was studied in detail in Chapter 4 and it was shown that the antigen is not expressed until the oocysts have begun excystation (see Chapter 4). Additionally, intense fluorescence was observed for several other developmental stages.

Therefore, the aim of this study was to examine the interaction between *Cryptosporidium* and biofilms by labelling *Cryptosporidium* developing stages from a biofilm with Sporo-Glo™ antibody and examining them using a confocal laser scanning microscope over a 6 day period.
7.2 Materials and Methods

7.2.1 Cryptosporidium and Pseudomonas Preparation

Cryptosporidium oocysts and Pseudomonas bacteria were prepared as described in the Materials and Methods section of Chapter 2 (Sections 2.2.2 and 2.2.1).

7.2.2 Cryptosporidium-exposed Pseudomonas aeruginosa Biofilm System

The Cryptosporidium-exposed biofilm systems were set up for 1, 3 and 6 days and biofilm dispersion was then performed as described in Chapter 2 (Sections 2.2.3 and 2.2.4).

7.2.3 Immunolabelling

7.2.3.1 FITC-Sporo-Glo™ Antibody

Initially, an aliquot of living dispersed biofilm suspension (20 µL) was immunolabeled with FITC-Sporo-Glo™ antibody as described in Chapter 4 (Section 4.2.7.1). Due to photobleaching issues, the dispersed biofilms were labelled with quantum-dot conjugated Sporo-Glo™ antibody as described in Chapter 4 (Section 4.2.7.2). The specificity and the binding pattern of Sporo-Glo™ antibody have been examined in Chapters 3 and 4. The binding pattern of FITC-Sporo-Glo™ and quantum dots-conjugated Sporo-Glo™ were compared and shown to be identical (Chapter 4), hence the observations of Cryptosporidium provided by these two
fluorescent antibodies were used to described developmental stages in the current experiments.

7.2.4 Confocal Microscopy Examination

The labelled biofilms were examined under a Leica SP2 confocal laser microscope as described in Chapter 4 (Section 4.2.7.2). Bright field images were also taken of Cryptosporidium cells that could be observed on the surface of dispersed biofilms.

7.3 Results

7.3.1 Day 1

When unexcysted oocysts were introduced into the 1 day-old immature biofilm system, induction of the excystation process within biofilms was observed to occur at variable rates. No fluorescence was detected in these trapped oocysts, indicating that these oocysts may be unexcysted or had already excysted (Figure 7.1). However, some free sporozoites with rounded posterior ends that tapered to a pointed anterior end were detected (Figure 7.2). According to the description by Petry et al. (2009), the presence of these free elongated fluorescent sporozoites (1.0 x 3.5 μm) suggests that they were derived from oocysts that excysted any time up to 24 hr prior to observation. No other developmental stages were fluorescently labelled and detected in 1 day-old Cryptosporidium-exposed biofilms.
Figure 7.1: *Cryptosporidium* oocysts identified within 1 day-old *Cryptosporidium*-exposed dispersed biofilms. Both confocal (A) and bright field (B) images show oocysts in the same location. Squares indicate the position of oocysts in the bright field images. Scale bars = 5 µm.

Figure 7.2: *Cryptosporidium* sporozoites within 1 day-old *Cryptosporidium*-exposed biofilms. Scale bar = 1 µm.
7.3.2 Day 3

After 3 days, fluorescent oocysts (Figure 7.3.A) and slender free sporozoites with a rounded posterior and a pointed tapered anterior end were routinely observed (Figure 7.3.C). Furthermore, some sporozoites were rounding up (1.5 x 3.0 µm; Figure 7.4.A) with less prominent anterior and posterior ends, which complements the previous description of morphological changes to sporozoites during the trophozoite transformation process (Current and Reese, 1986). Therefore, several well formed, individual oval shaped cells were believed to be trophozoites (Figure 7.4.B; 1.5 x 3.0 µm). These trophozoites closely resembled those seen in the study by Hijjawi et al. (2004). It is assumed that variable number trophozoites fused by the syzygy process (Figure 7.5) to form type I meronts. This study showed that the side to side syzygy strategy was adopted for aggregates of 2 to 3 trophozoites while in aggregates of more than 4 trophozoites, both side to side and head to head strategies were used (Figures 7.5 and 7.6).
Figure 7.3: *Cryptosporidium* excysted oocysts and sporozoites identified within 3 day-old *Cryptosporidium*-exposed biofilms. Fluorescent expression from oocysts (A) and sporozoites (A & C) is indicative of the excystation process. B) Bright field image of the oocyst as seen in A. Arrowhead indicates sporozoites and arrow indicates oocysts. Scale bars = A & B: 5 µm; C: 3 µm.
Figure 7.4: Cryptosporidium sporozoites and trophozoites identified within 3 day-old Cryptosporidium-exposed biofilms. A) Tapered sporozoites. B) Trophozoites. Scale bars = 3 µm.
Figure 7.5: *Cryptosporidium* trophozoites identified within 3 day-old *Cryptosporidium*-exposed biofilms. A-D) Aggregations of trophozoites. Scale bars = A: 5 µm; B: 3 µm; C & D: 5 µm.
7.3.3 Day 6

On day 6, excysting oocysts and free sporozoites continued to be detected (Figures 7.7 and 7.8). Similar to previous observations made in 1 and 3 day-old biofilms, newly released sporozoites (< 24 h) were slender and curved with a pointed end (4.0 x 0.5 μm; Figure 7.7). In addition, some sporozoites were also observed to round up.
gradually to give the appearance of comma shaped sporozoites with a distinctive rounded posterior end and a tapering body (3.0 x 2.5 µm; Figure 7.8), indicating that they were in the process of forming trophozoites. With this, oval shaped trophozoites were observed (1.5 x 2.0 µm; Figure 7.9.A & B), sometimes fusing with other trophozoites via the syzygy process (Figure 7.9.C) to form, presumably, type I meronts.

Figure 7.7: Cryptosporidium oocysts and sporozoites identified within 6 day-old Cryptosporidium-exposed biofilms. Image shows an excysting oocyst (arrow head) and the slender shaped sporozoite (arrow). Scale bar = 10 µm.

Figure 7.8: Cryptosporidium sporozoites identified within 6 day-old Cryptosporidium-exposed biofilms. Scale bars = 2.5 µm
Two morphologically different meronts similar to those observed by Hijawi et al. (2004) were observed (Figures 7.10 and 7.11), confirming that the merogony process had occurred within biofilms. According to the descriptions by Hijawi et al. (2004), the gamonts that appear as grape-like aggregates were presumed to be type I meronts (Figure 7.10.A & B). These aggregates were formed by many small circular to oval shaped type I merozoites (0.5 x 0.5 μm). Once matured, type I merozoites were released (1.0 x 1.0 μm; Figure 7.10.C). The gamonts with variable aggregate patterns
belonged to type II meronts (Figure 7.11). These meronts were formed by variable numbers of spindle shaped cells with pointed ends, which are type II merozoites (0.25 x 0.5 µm).

Previous studies (Current and Reese, 1986; Hijjawi et al., 2004) have shown that microgamonts are easily distinguished from macrogamonts by the presence of a large number of developing microgametes. Hence, in this study, it was presumed that type II merozoites underwent binary fission during microgamont development (Figure 7.12.A). In addition, these small circular shaped (2.0 x 2.0 µm) gamonts, with several still-developing zoites, resembled immature microgamonts as observed in the study by Current and Reese (1986). Furthermore, as microgamonts matured (5.0 x 5.0 µm), they contained masses of microgametes (Figure 7.12.B). Consistent with the descriptions by Current and Reese (1986), the observation of free bullet shaped zoites (0.25 x 0.30 µm) within biofilms belonged to microgametes that had budded off from microgamonts (Figure 7.13).

At the same time, gamonts that were oval in shape and similar in size to oocysts (2.5 x 3.0 µm) were presumed to be macrogamonts (Figures 7.12.C). The morphology of these macrogamonts were consistent with the macrogamonts observations made by Hijjawi et al. (2004) and were intensely labelled. This degree of fluorescence intensity was never observed in either excysted or unexcysted oocysts. Furthermore, as shown in Chapter 4, excysted oocysts that were labelled with Sporo-Glo™ antibody, revealed the structure of internal sporozoites, which were not seen here.

Interestingly, stage 2 gamont-like cells were observed within the 6 day-old biofilms (Figure 7.14). This gamont-like stage in biofilms was also blunt-ended and rod-shaped (2.5 x 5.0 µm), which is complemented by the previous description of
Hijjawi et al. (2004). The role of this stage in the Cryptosporidium life cycle is unknown but based upon the observation of superimposed confocal and brightfield images (Figure 7.14.C), this stage may play a role in producing merozoites or trophozoites, as these internal structures were intensely labelled by Sporo-Glo™ antibody.

Figure 7.10: Cryptosporidium type I meronts and type I merozoites identified within 6 day-old Cryptosporidium-exposed biofilms. A & B) Grape-like structure of type I meronts. C) Free type I merozoites. Scale bars = A & B: 5 µm; C: 1 µm.
Figure 7.11: *Cryptosporidium* type II meronts identified within 6 day-old *Cryptosporidium*-exposed biofilms. Scale bars = A, B, D: 5 µm; C: 3 µm.
Figure 7.12: Cryptosporidium micro- and macro-gamonts identified within 6 day-old Cryptosporidium-exposed biofilms. A) Immature microgamont that has undergone the binary fission process. B) Mature/developing microgamont. Arrows indicated microgametes. C) Macrogamont. Scale bars = A & B: 2 µm; C: 2.5 µm.
Figure 7.13: *Cryptosporidium* microgametes identified within 6 day-old *Cryptosporidium*-exposed biofilms. Scale bars = 0.5 µm.
Figure 7.14: Cryptosporidium stage II gamont-like cell identified within 6 day-old Cryptosporidium-exposed biofilms. A & B) Confocal and brightfield images of the gamont-like cell. C) Superimposed image of confocal and bright field images. Scale bars = 2.5 µm.

7.4 Discussion

The findings described here demonstrate that Cryptosporidium not only can excyst within aquatic biofilms, but also shows that they can multiply extracellularly without the requirement of host encapsulation. In this study, Cryptosporidium began their excystation process at day 1. By the third day, the series changes in sporozoite
morphologies from the slender shaped to the more oval shaped sporozoites was observed. Although previous studies by Petry et al. (2009) and Matsubayashi et al. (2010) suggested these changes were due to aged sporozoites that could not multiply in the nutrient limited, cell-free culture environment, the observation of subsequent Cryptosporidium developmental stages within 6 day-old biofilms demonstrated that these sporozoites were not simply aged sporozoites. In addition, similar morphological changes to sporozoites during the trophozoite transformation process have been previously reported (Tyzzer, 1910; Levine, 1985; Current and Reese, 1986). Furthermore, the environments in which the liberated sporozoites are exposed to in aquatic biofilms are nutrient rich micro-environments that would allow Cryptosporidium to salvage their metabolic needs to fuel their high rate of growth and multiplication. Therefore, it is unlikely that the observations made from this study are due to aged sporozoites.

By day 6, developmental stages of Cryptosporidium were much more prominent and easily identified. Other developmental stages included type I/II meronts, type I/II merozoites, micro-/macro- gamonts, and the gamont-like stages. In addition, this study indicates the life cycle of Cryptosporidium in biofilms involves both multiple and binary fission. Multiple fission is commonly used by Cryptosporidium (Thompson et al., 2005). It is initiated when the nucleus of the parent cell divides several times by mitosis to generate multiple nuclei. Following this, the cytoplasm is separated to create multiple daughter cells (Poyton, 1973). Stages at which this process occurs include merogony (to produce merozoites), gametogony (to produce gametes) and sporogony (to produce sporozoites). In contrast, binary fission is a process in which an organism duplicates its DNA and then divides into two parts with each new organism receiving the same copy of DNA (Angert, 2005). The
*Cryptosporidium* binary fission process is rarely reported (Borowski *et al.*, 2010); therefore, the observation of the binary fission process during microgamont development in this study requires further investigation.

Similar to other cell-free cultures (Hijjawi *et al.*, 2004; Boxell *et al.*, 2008), fused trophozoites were commonly observed in the biofilms. These trophozoites may have fused together by the syzygy process for the purpose of increasing *Cryptosporidium* genetic diversity in the absence of a host. These fused trophozoites could not just be due to the trophozoites coincidentally clumping together after dispersion. This is because nitric oxide simply is used to detach biofilms from the flow cell surface (Barraud *et al.*, 2009), it will not segregate bacteria or microbes within biofilms into individual cells. Syzygy (side to side/head to head pairing) is also commonly used by extracellular gregarine trophozoites (unattached to epithelial cells) and many studies have shown that *Cryptosporidium* is closely related to gregarines either phylogenetically or morphologically (Carreno *et al.*, 1999; Hijjawi *et al.*, 2004; Barta and Thompson, 2006; Boxell *et al.*, 2008; Valigurová *et al.*, 2008; Borowski *et al.*, 2010; Hijjawi *et al.*, 2010). As such, similar to gregarines, the *Cryptosporidium* syzygy process appears to be more commonly observed in the absence of a host, such as in cell-free cultures or in biofilms as compared to *in-vivo* (Current and Reese, 1986) or *in-vitro* (Borowski *et al.*, 2010) culture. In addition, similar to the observations made in cell-free culture (Hijjawi *et al.*, 2004; Boxell *et al.*, 2008), the aggregate of meronts as observed in this study also most likely arose from the multiple mitotic divisions of these fused trophozoites; hence, meronts of variable sizes were observed.

Previously reported (Hijjawi *et al.*, 2002; Hijjawi *et al.*, 2004) stage II gamont-like cells were also identified in this study. This stage has not been commonly observed
in the *Cryptosporidium* life cycle, with the study by Woods and Upton (2007) suspecting the presence of this stage in both cell-free and *in-vitro* cultures was due to contaminating debris and fungal infection, resembling *Bipolaris australiensis* and *Colletotrichum acutatum*. However, the intense fluorescence signal of the internal structures, which resembled merozoites or trophozoites, disagrees with this argument. As compared to *in-vitro* culture and *in-vivo* (Current and Reese, 1986; Borowski *et al*., 2010), this stage is more commonly observed in cell-free culture (Hijjawi *et al*., 2004; Hijjawi *et al*., 2010), hence together with the observations made in this study, it may suggest that the role of this stage is to generate trophozoites and merozoites so that more new oocysts can be produced in the absence of a host.

In conclusion, these novel findings not only show that *Cryptosporidium* can multiply extracellularly within a biofilm environment, but that they also have significant implication for the biology and classification of *Cryptosporidium*.

### 7.5 References


Morphological Changes of *Cryptosporidium* after Exposure to Aquatic Biofilms: A Scanning Electron Microscopy Study
8.1 Introduction

Detection and visualisation of *Cryptosporidium* in cell-free, *in-vitro* cultures and *in-vivo* not only provides more detailed information that often cannot be achieved by molecular techniques, but also accelerates the understanding of survival strategies adopted by *Cryptosporidium* when they are exposed to different environments (Hijjawi *et al.*, 2004; Karanis *et al.*, 2008; Valigurová *et al.*, 2008; Borowski *et al.*, 2010; Edwards *et al.*, 2012). Through the use of microscopy, it is now understood that *Cryptosporidium* is not an obligate intracellular microorganism but is, in fact, both an epicellular and extracellular parasite (Upton *et al.*, 1995; Valigurová *et al.*, 2007; Borowski *et al.*, 2010; Hijjawi *et al.*, 2010).

Currently, the fastest and most accurate way to examine *Cryptosporidium* is through a combination of immunolabelling and confocal/epifluorescent microscopy (Boxell *et al.*, 2008; Zhang *et al.*, 2009; Hijjawi *et al.*, 2010). Immunolabelling and confocal/epifluorescent microscopy is a non-destructive method and does not involve complicated sample processing. This method has successfully revealed *Cryptosporidium* behaviour in cultures such as biofilms (Chapter 7), cell-free and *in-vitro* cultures (Carreno *et al.*, 2001; Boxell *et al.*, 2008; Zhang *et al.*, 2009; Hijjawi *et al.*, 2010; Lalancette *et al.*, 2010). However, this method provides limited micro-structural information on *Cryptosporidium* and lacks the ability to provide high resolution imaging, especially of sub-micron cells such as merozoites and microgametocytes. Therefore, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) have gained popularity in this field of study (Valigurová *et al.*, 2007; Jirku *et al.*, 2008; Valigurová *et al.*, 2008; Borowski *et al.*, 2010). SEM and TEM have greater resolution compared to confocal microscopy and has provided
new insights into Cryptosporidium structural changes in both in-vitro culture and in-vivo (Valigurová et al., 2007; Valigurová et al., 2008; Borowski et al., 2010; Edwards et al., 2012).

To gain more detailed information about the morphological changes of Cryptosporidium after exposure to biofilms, SEM was used in this study. To show that the observed developmental stages belong to Cryptosporidium, correlation between SEM and confocal microscopic observations was performed. Detailed structural observations made from SEM alone and SEM in combination with confocal microscopy, can further provide new perspectives and additional insights into the extent of extracellular multiplication of Cryptosporidium and also the importance of the surrounding environment on shaping the life cycle of Cryptosporidium.

8.2 Materials and Methods

8.2.1 Pseudomonas aeruginosa and Cryptosporidium Preparation

Pseudomonas aeruginosa bacteria and Cryptosporidium oocysts were prepared as described in the Materials and Methods section of Chapter 2 (Sections 2.2.1 and 2.2.2).
8.2.2 Flow Cell Biofilm System

One, three and six day-old Cryptosporidium-exposed biofilm systems were set up as described in Chapter 2 (Section 2.2.3).

8.2.3 Biofilm Dispersion

Biofilm dispersion was performed and the suspension was collected and fixed with 2.5% paraformaldehyde as described in Chapters 2 (Section 2.2.4).

8.2.4 Scanning Electron Microscopy

Prior to SEM examination, an aliquot (20 µL) of fixed flow cell biofilm sample was prepared as described in Chapter 4 (Section 4.2.8.3).

8.2.5 Correlative Confocal Microscopy with Scanning Electron Microscopy

8.2.5.1 Immunolabelling

For the correlation study, the biofilm suspensions were immunolabeled with quantum dot conjugated Sporo-Glo™ antibody as described in Chapter 4 (Section 4.2.7.2).
8.2.5.2 Correlative Observation

To confirm the relationship of observations made with SEM to Cryptosporidium, correlation of SEM with confocal microscopy was performed. The correlation study was carried out by either correlative method 1 or 2 as described in Chapter 4 (Section 4.2.9). For meronts, serial sections on the xy plane were obtained at 0.44 µm along the z-axis and the three dimensional Z-stack images were constructed using Volview.

8.2.5.3 Confocal Microscopy

Confocal microscopy examination was performed as described in Chapter 4 (Section 4.2.7.2) and the location of relevant cells on the SEM finder grid pattern was recorded.

8.2.6 Ultra-thin Sectioning and Imaging

During SEM observations, unknown extra large gamont-like cells (>50 µm) were detected. To further determine the role of these cells optical sectioning was attempted to locate these cells. Biofilms were dispersed before being fixed and stored in 2.5% glutaraldehyde in sterile 1 x PBS. Samples were subsequently processed in a microwave (PELCO, Biowave) by post-fixing in 1% OsO₄ in 0.1 M PBS, dehydrating through a graded series of ethanol and acetone, and infiltrating and embedding in Procure Araldite epoxy resin, before being cured at 60°C for 24 h.
Sections 1 µm-thick were cut with glass knives, mounted on glass slides and stained with toluidine blue. Images were acquired on a Zeiss Axioskop optical microscope fitted with an Axiovision digital camera.

8.3 Results

8.3.1 The Association of Cryptosporidium with 1 Day-old Biofilms

On day 1, oocysts appeared to be engulfed by the biofilms’ polysaccharide matrix (Figure 8.1). In addition, gamonts with a prominent radial fold and a button-like dense band that resembled the parasitophorous vacuole (PV) were detected in this experiment (Figure 8.2), indicating that Cryptosporidium development had occurred.

![Figure 8.1](image)

Figure 8.1: The retention of oocysts (arrowhead) within 1 day-old biofilms. Arrow indicates biofilm matrix. Triangle indicates bacteria. Scale bar = 1 µm.
Figure 8.2: A) Evidence of a parasitophorous vacuole. B) Magnified region depicted in A. Arrow indicates radial fold and arrowhead indicates dense band area. Scale bars = A: 1 µm; B: 200 nm.
8.3.2 The Association of Cryptosporidium with 3 Day-old Biofilms

Within 3 day-old biofilms, variable-shaped individual sporozoites and trophozoites were observed, indicating that Cryptosporidium were undergoing trophozoite transformation (Figure 8.3). These include comma shaped sporozoites (Figure 8.3.A) and rod shaped trophozoites (1.0 x 2.0 µm; Figure 8.3.B). Occasionally, bell shaped trophozoites (Figure 8.3.C) were identified, these may have resulted in the formation of circular shaped trophozoites seen in 6 day-old biofilms (Figure 8.6.B).
Figure 8.3: Example of the trophozoite transformation process. Comma shaped sporozoite (A) transformed into either rod shaped (B) or bell shaped (C) trophozoites.

Scale bars = A: 2.5 µm; B: 1 µm; C = 0.5 µm.
8.3.3 The Association of Cryptosporidium with 6 Day-old Biofilms

As more unexcysted oocysts were introduced into the system, detailed morphological changes to unexcysted oocysts were easily identified. It was observed that biofilm associated Cryptosporidium also released sporozoites by creating a cleft-like opening in the oocysts (Figure 8.4.A). An empty oocyst with a wide opening and rough membrane was observed (Figure 8.4.B). Furthermore, the identification of perforated oocysts (Figure 8.4.C & D) suggested that these degraded oocysts may be formed by the oocysts that had been excysted on day 1 or day 3.

![Figure 8.4: Empty oocysts identified within 6 day-old biofilms. A) Sporozoites released via a cleft-like opening. B) Empty oocysts with a rough membrane appearance. C & D) Degraded excysted oocysts. Scale bars = A: 2 µm; B: 1 µm; C: 1 µm; D: 4 µm.](image)

Free sporozoites (Figures 8.5), trophozoites (Figure 8.6), and PVs (Figure 8.7) were observed. The PVs observed within 6 day-old biofilms were similar to that seen in day 1 (Figure 8.2) in that it had a distinctive radial fold and button-like base. Several
large gamont-like cells (> 10 µm; Figure 8.8) similar in size to type I and II merozoits that had been examined under confocal microscopy (Chapter 7: Figures 7.10 & 7.11) were observed. These gamonts were further confirmed to be meronts when several unreleased type II merozoites were detected within a single type II meront (Figure 8.8.E). Free type I (circular shaped; 0.7 x 0.7 µm; Figure 8.9.A & B) and II merozoites (spindle shaped; 0.5 x 1.5 µm; Figure 8.9.C) were also identified.

The microgamonts within biofilms were also large (~15µm; Figure 8.10) and contained a large number of microgametes. Although these microgametes appeared to bud off from the microgamont, the identity of sausage-like structure on the surface of microgamonts could not be determined. In addition, multiple unknown extra large gamont-like cells were detected (> 50 µm; Figure 8.11).

Figure 8.5: Free sporozoite identified within 6 day-old biofilms. Scale bar = 2 µm
Figure 8.6: Rod (A)/circular shaped (B) trophozoites. Scale bars = A: 500 nm; B: 1 µm.
Figure 8.7: A) Parasitophorous vacuole. B) Magnified area depicted in A. Arrow indicates radial fold and arrowhead indicates dense band area. Scale bars = A: 2 µm; B: 1 µm.
Figure 8.8: Large gamont cells (meronts) identified within 6 day-old biofilms (A-D). E) type II meront with some type II merozoites (circled) within. Scale bars = A: 5 µm; B: 4 µm; C: 2.5 µm; D: 4 µm; E: 2 µm.
Figure 8.9: Type I (A & B) and type II (C) merozoites identified within 6 day-old biofilms. Scale bars = A & B: 1 µm; C: 500 nm
Figure 8.10: A) Microgametes identified within 6 day-old biofilm; B) magnified area depicted in image A. Scale bars = A: 5 µm; B: 2 µm.
Figure 8.11: Extra large gamont-like cells identified within 6 day-old biofilms. Scale bars = 10 µm.

8.3.4 Ultra-thin Sectioning and Imaging

Due to the immense numbers of bacteria relative to *Cryptosporidium*, several attempts at cutting thin sections for transmission electron microscopy failed to locate the extra-large gamont cells within the mass of biofilm. However, throughout the ultra thin sectioning process, meronts (Figure 8.12) that had similar cell sizes to those observed with SEM (Figure 8.8) were identified.
Figure 8.12: Ultra-thin sectioning images of large gamont-like cells (meronts) within 6 day-old biofilms. Scale bars = A: 5 µm; B: 10 µm.
8.3.5 Correlative Studies

Correlative studies using confocal and SEM confirmed that the observed extracellular stages in 6 day-old Cryptosporidium-exposed biofilms belonged to Cryptosporidium. Several key developmental stages representing both asexual and sexual reproduction were successfully correlated, including sporozoites that resided within oocysts, trophozoites, meronts and merozoites (Figures 8.13-8.15).

Figure 8.13 (A & B) showed that some sporozoites had already escaped into the external environment. Hence, when this oocyst was further processed for SEM analysis, a collapsed cell was observed, indicating this oocyst was no longer as compact as unexcysted oocysts. In addition, SEM confirmed structures that intensely expressed Sporo-Glo™ antibody when examined under confocal microscopy were trophozoites and merozoites (Figure 8.13.C & D; Figure 8.14.A & B).

Under confocal microscopy, a group of type I merozoites were detected (Figure 8.14.D). However, SEM determined that this group of cells were not free merozoites but rather that they were residing within a meront that was not fluorescently labelled by Sporo-Glo™ antibody (Figure 8.14.C) and therefore not visible in the confocal image. Throughout the study, confocal microscopy revealed several membrane-bound cellular structures (Figures 8.15.A). Both the membrane and internal structures were fluorescently labelled. Similar to the observation made in Movie 8.1, when these cells were observed under SEM, an undulated contour membrane structure was observed (Figure 8.15.B & C). Based upon the cell size (12 x 12 μm), these cells were presumed to be meronts.
Figure 8.13: The correlation of excysted oocyst (A & B) and trophozoite (C & D) by SEM (A & C) and confocal microscopy (B & D). Scale bars = A: 1 µm; B: 2 µm; C: 500 nm; D: 1 µm.
Figure 8.14: The correlation of free type II merozoites (A & B) and a type I meront containing type I merozoites (C & D) under SEM (A & C) and confocal microscopy (B & D). Scale bars = A: 200 nm; B: 500 nm; C & D: 1 μm.
8.4 Discussion

This study confirms the fluorescent labelling of *Cryptosporidium* detected by confocal microscopy (Chapter 7) was not due to degenerate or accumulated oocysts. In addition, SEM not only revealed detailed surface structural data for several stages, such as sporozoites, trophozoites, free and encapsulated type II merozoites, type I
and II meronts, and microgamonts, but also demonstrates that *Cryptosporidium* has the capability to form a PV independent of a host.

Similar to other reports of *Cryptosporidium* excystation (Reduker *et al.*, 1985; Borowski *et al.*, 2008; Borowski *et al.*, 2010), *Cryptosporidium* sporozoites in biofilms were also released from *Cryptosporidium* oocysts by creating a cleft-like opening at the suture. This hence allowed their subsequent development through the formation of a PV. The formation of a PV within biofilms is intriguing as PV has not previously been reported in cell-free cultures (Hijjawi *et al.*, 2004; Zhang *et al.*, 2009; Hijjawi *et al.*, 2010). This may be due to the fact that it is difficult to identify PVs using optical microscopy and hence the possibility of forming a PV in the cell-free culture was not considered. However, this study provides evidence that *Cryptosporidium* extracellular behaviour may involve the formation a PV and their morphologies greatly resemble PVs that are formed in *in-vitro* cultures (Figure 8.16; Borowski, personal communication). Furthermore, the PV observed here were very similar to cells observed in previous *in-vitro* and *in-vivo* studies (Current and Reese, 1986; Landsberg and Paperna, 1986; Rosales *et al.*, 1998; Valigurová *et al.*, 2007; Valigurová *et al.*, 2008) where it was believed that the undulated membrane was part of the PV membrane.
The origin of the PV is contentious and there are two theories about PV formation. One theory is that the PV is derived from the host, not the parasite itself (Lumb et al., 1988; Bonnin et al., 1999; Elliott and Clark, 2000; Huang et al., 2004; Valigurová et al., 2007), while the second theory is that Cryptosporidium does not require a host cell to form its own PV because the PV is produced by
Cryptosporidium (Pohlenz et al., 1978; Gjerde, 1986; Edwards et al., 2012). This study conforms with the latter theory and also agrees that the PVs of different stages of Cryptosporidium have different structures and morphologies (Beyer et al., 2002). As such, different surface epitopes may have been expressed, which would explain why not all PVs were fluorescently labelled by Sporo-Glo™.

This study suggests that Cryptosporidium can form a PV independently and its function may be similar to the PV formed in the host. It is a survival strategy of Cryptosporidium to compete for nutrients with other bacteria residing within the same microcolony, and also forms a protective boundary between Cryptosporidium and bacteria. The formation of a PV would allow more efficient nutrient acquisition within the crowded and competitive environment as compared to using passive and facilitated diffusion (Bannister, 1979). As such, favourable conditions are created inside the PV for development and reproduction purposes. In addition, it is probable that PVs can be used as protective barrier from bacterial digestion, predators and quorum sensing. Furthermore, the formation of a PV may provide space for Cryptosporidium to multiply, hence the vacuole is likely to expand and change as the parasite grows and multiplies. Therefore, this may explain why variable PV shapes were observed in this study.

Besides PV formation, the identification of large meronts (Figure 8.15) and extra large gamont-like stages (Figure 8.11) suggests that biofilms can modify and influence Cryptosporidium developmental stages in aquatic environments. Although the identity and the role of extra large gamont-like cells are still unknown, similar cell sizes and morphologies have been observed in gregarines (Leander, 2006; Leander, 2007; Alarcón et al., 2011). Therefore, it is suspected that they may also have a role in producing infective stages of Cryptosporidium in biofilms. Although
these stages were not labelled by Sporo-Glo™ antibody, it does not mean they were not *Cryptosporidium*. For example, no similar cells were observed from biofilm-only controls. In addition, the correlative imaging of a large meront showed that apart from type I merozoites located within the cell producing fluorescence, no other part of the parent cell fluoresced. The inability to label these gamont stages and meront walls with Sporo-Glo™ antibody suggests surface epitopes that bind Sporo-Glo™ were not expressed by these stages in the biofilms.

In conclusion, this study not only shows that biofilms can significantly influence, support and shape the life cycle of *Cryptosporidium* extracellularly, but also highlights the importance of using appropriate fluorescent antibodies. The observations made in this study suggests that Sporo-Glo™ antibody does not label all extracellular stages in biofilms and a new antibody that targets more of the *Cryptosporidium* developmental stages is needed to further investigate the life style of *Cryptosporidium* in biofilms. Knowing what the antibody binds to would also help to clarify this. To date, however, no relevant data is available.

### 8.5 References


9

General Discussion
This study has demonstrated, both qualitatively and quantitatively, that *Cryptosporidium* can multiply extracellularly in biofilms. Despite the fact that the capability of *Cryptosporidium* to multiply extracellularly remains controversial, there is increasing evidence to suggest that their extracellular capability cannot be disregarded (Angles *et al.*, 2007). Water is a main route of exposure to *Cryptosporidium* and the association of *Cryptosporidium* with biofilms is believed to be responsible for episodes of sporadic *Cryptosporidium* waterborne outbreaks (Howe *et al.*, 2002). However, as *Cryptosporidium* was not believed to have the capability to proliferate within biofilms (Holt, 1995), previous studies focused only on the “oocysts” stage (Keevil, 2003; Searcy *et al.*, 2006; Wolyniak *et al.*, 2009, 2010). This study, therefore, focused on investigating the subsequent fate of oocysts after capture by biofilms using a combination of correlative techniques, including analytical qPCR (Chapter 5), flow cytometry (Chapter 6), confocal microscopy (Chapter 7) and SEM (Chapter 8). The results from all of these analyses corroborate the hypothesis that biofilms have the ability to support *Cryptosporidium* development in aquatic biofilms.

Overall, *Cryptosporidium* life cycles in biofilms (Figure 9.1; Chapters 7 & 8) is generally similar to their life cycle in *in-vitro* (Borowski *et al.*, 2010), cell-free cultures (Hijjawi *et al.*, 2004) and *in-vivo* (Current and Reese, 1986), including the generation of sporozoites, trophozoites, type I/II meronts, type I/II merozoites, and micro- and macro-gamonts. In addition, when the efficacy of *in-vitro* (Borowski *et al.*, 2010), cell-free cultures (Hijjawi *et al.*, 2004), *in-vivo* (Current and Reese, 1986), biofilms and biofilm-free systems in supporting *Cryptosporidium* development were compared (Table 9.1), *in-vivo* provides the best growth conditions because oocysts excysted within 4 hr, and all developmental stages were detected within 48 hr.
(Current and Reese, 1986). Despite *Cryptosporidium* being able to complete their life cycle within a 4-6 day period in *in-vitro* (Borowski *et al*., 2010) and cell-free cultures (Hijjawi *et al*., 2004), unlike biofilm system, development of *Cryptosporidium* does not occur without *in-vitro* excystation through acid treatment.

Figure 9.1: The proposed life cycle of *Cryptosporidium* in biofilms.
Table 9.1: Comparison of the efficacy of *Cryptosporidium* development at different environments. Data from present study and other (Current and Reese, 1986; Hijjawi *et al.*, 2004; Edwards *et al.*, 2012)

<table>
<thead>
<tr>
<th>In-vitro Excystation</th>
<th>Biofilm System</th>
<th>Biofilm-free System</th>
<th>In-vivo</th>
<th>In-vitro</th>
<th>Cell-free</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sporozoites</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Trophozoites</td>
<td>&gt; 24 h</td>
<td>No</td>
<td>&gt; 4 h</td>
<td>&gt; 3 h</td>
<td>&lt; 24 h</td>
</tr>
<tr>
<td>Type I Meronts</td>
<td>&gt; 144 h</td>
<td>No</td>
<td>&gt; 16 h</td>
<td>&gt; 24 h</td>
<td>&gt; 48 h</td>
</tr>
<tr>
<td>Type I Merozoites</td>
<td>&gt; 144 h</td>
<td>No</td>
<td>&gt; 20 h</td>
<td>&gt; 24 h</td>
<td>&gt; 48 h</td>
</tr>
<tr>
<td>Type II Meronts</td>
<td>&gt; 144 h</td>
<td>No</td>
<td>&gt; 24 h</td>
<td>&gt; 72 h</td>
<td>&gt; 48 h</td>
</tr>
<tr>
<td>Type II Merozoites</td>
<td>&gt; 144 h</td>
<td>No</td>
<td>&gt; 24 h</td>
<td>&gt; 72 h</td>
<td>&gt; 144 h</td>
</tr>
<tr>
<td>Microgamonts</td>
<td>&gt; 144 h</td>
<td>No</td>
<td>&gt; 36 h</td>
<td>&gt; 96 h</td>
<td>&gt; 144 h</td>
</tr>
<tr>
<td>Macrogamonts</td>
<td>&gt; 144 h</td>
<td>No</td>
<td>&gt; 48 h</td>
<td>&gt; 96 h</td>
<td>&gt; 144 h</td>
</tr>
</tbody>
</table>
The capacity of *Cryptosporidium* to multiply both intracellularly and extracellularly further reflects the fact that it is closely related to gregarine protozoa (Carreno *et al.*, 1999; Hijjawi *et al.*, 2002; Rosales *et al.*, 2005), which can also multiply by either means (Leander *et al.*, 2003). Furthermore, it was observed the life cycle of *Cryptosporidium* is similar to that observed in the gregarine life cycle (Rueckert *et al.*, 2011), which also has variable morphotypes in different environments for survival purposes (Table 9.2). Thus, several unique morphotypes or mechanisms (Chapters 5 - 8) that have never been observed in *in-vitro* (Borowski *et al.*, 2010), cell-free cultures (Hijjawi *et al.*, 2004) or *in-vivo* (Current and Reese, 1986) were identified, including forming a PV independently (Figures 8.2), large meronts (Figures 8.8 and 8.15), mass production of merozoites (Figures 7.10 and 7.11) and microgametes (Figures 7.12.B and 8.10), and extra large gamonts (Figure 8.11).

Since the proposal that *Cryptosporidium* can form a PV independently was made by Pohlenz *et al.* (1978), this is the first time that a PV has been shown to form in a host free system or biofilm (Figure 8.2). This study has identified similar PV structures such as radial folds, which may represent feeder cell organelles; and button like bases, which are equivalent to the dense bands seen in TEM, as observed in other *in-vitro* and *in-vivo* studies (Valigurová *et al.*, 2008; Borowski *et al.*, 2010). Furthermore, meronts in biofilms (Figure 8.15 and Movie 8.1) also had undulated contour membranes (PV membranes), that have also been observed *in-vivo* (Landsberg and Paperna, 1986; Paperna, 1987). These authors believed this had been derived from the host microvillus, yet no host was present in the current study, leading to the belief that the extracellular multiplication of *Cryptosporidium* may involve the self-formation of a PV for survival, and as a strategy to concentrate nutrients from their surrounding environment.
In addition, trophozoites in biofilms aggregated (Figure 7.5) in a way that was similar to the gregarine syzygy process (Leander, 2008). Different species of gregarine usually have different orientations during the syzygy process, which can either be head to head or side to side (Leander, 2008). Therefore, it was initially believed that the *Cryptosporidium* syzygy process was always head to head as previously observed in *in-vitro* and *in-vivo* studies (Valigurová *et al.*, 2008; Borowski *et al.*, 2010; Edwards *et al.*, 2012). Moreover, the aggregation of trophozoites/merozoites is generally considered to involve aged sporozoites that tend to aggregate in the absence of a suitable host (Petry *et al.*, 2009; Matsubayashi *et al.*, 2010). Nevertheless, the subsequent development of *Cryptosporidium*, and the formation of meront walls (PV membrane) around aggregated merozoites were observed (Figures 8.14.C & D; 8.15), which further implies that these aggregated merozoites (Figures 7.10 and 7.11) were not formed from aged sporozoites, as previously hypothesised by Petry *et al.* (2009).

Interestingly, both meronts and microgamonts in biofilms (Figures 7.10 and 7.11; 8.8, 8.10 and 8.15) were much larger (> 10 µm) than those (~ 5 µm) observed in cell-free (Hijjawi *et al.*, 2004), *in-vitro* cultures (Valigurová *et al.*, 2008; Borowski *et al.*, 2010) and *in-vivo* (Current and Reese, 1986). Furthermore, type I and II merozoites produced within a single meront in biofilms were in larger numbers (Figures 7.10 and 7.11) when compared to those in cell-free cultures (Hijjawi *et al.*, 2004). This may be due to the biofilms constantly undergoing erosion thus they may also face the challenge of being washed away. Therefore, the mass production of merozoites may help them to increase the chance of sexual reproduction in the biofilms.
In addition to the previously described type II gamont-like stage (Figure 7.14) (Hijjawi et al., 2002; Hijjawi et al., 2004; Borowski et al., 2010), several unknown extra large gamont-like stages were observed (~50 µm; Figure 8.11). These features are most likely reflective of their close affinity to gregarines, for example *Selenidium* spp., which can also often reach sizes above 50 µm (Gunderson and Small, 1986; Wakeman and Leander, 2012). Furthermore, when comparing other features of the *Cryptosporidium* life cycle (*in-vitro*, cell-free cultures, *in-vivo* and biofilm systems) to the gregarine life cycle, these two genera are very similar (Table 9.3). As such, the current study further supports the proposition that *Cryptosporidium* should no longer be considered a coccidian parasite.
Table 9.2: Comparison of the *Cryptosporidium* life cycle in different environments. Data from the present study and others (Hijjawi et al., 2002; Hijjawi et al., 2004; Petry, 2004; Valigurová et al., 2008; Petry et al., 2009; Borowski et al., 2010; Hijjawi et al., 2010; Edwards et al., 2012; Valigurová, 2012)

<table>
<thead>
<tr>
<th>Stage</th>
<th>In-vivo</th>
<th>In-vitro</th>
<th>Cell-free</th>
<th>Biofilms</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Host induction</td>
<td>In-vitro excystation with acid water</td>
<td>In-vitro excystation with acid water</td>
<td>Biofilm induction</td>
</tr>
<tr>
<td>Sporozoites</td>
<td>Slender shaped with rounded posterior and a pointed tapered anterior end (1.0 x 5.0 µm)</td>
<td>Slender shaped with rounded posterior and a pointed tapered anterior end (1.0 x 5.0 µm)</td>
<td>Slender shaped with rounded posterior and a pointed tapered anterior end (1.0 x 5.0 µm)</td>
<td>Slender shaped with rounded posterior and a pointed tapered anterior end (1.0 x 5.0 µm)</td>
</tr>
<tr>
<td>Parasitophorous Vacuole</td>
<td>Yes with distinctive radial fold and button like base</td>
<td>Yes with distinctive radial fold and button like base</td>
<td>No</td>
<td>Yes with distinctive radial fold and button like base</td>
</tr>
<tr>
<td>Trophozoites</td>
<td>Oval (3.0 x 1.5 µm) or dumbbell shaped (2.0 to 3.0 µm)</td>
<td>Oval shaped (4.0 x 3.0 µm)</td>
<td>Oval shaped (2.0 x 1.0 µm)</td>
<td>Spherical shaped (2.3 x 2.0 µm) or dumbbell-shaped</td>
</tr>
<tr>
<td>Syngy</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Trophozoites Aggregation</td>
<td>No</td>
<td>Yes, but when the invasion of host cell fails</td>
<td>Yes, frequently</td>
<td>Yes, frequently</td>
</tr>
<tr>
<td>Type I Meronts</td>
<td>Spherical shaped (5.0 x 5.0 µm), bounded by PV membrane</td>
<td>Oval shaped, bounded by PV membrane (4.0 x 5.0 µm)</td>
<td>Golgi-like aggregates, unbounded by PV membrane (5.0 µm in diameter)</td>
<td>Variable shapes, mostly spherical (average 15.0 x 10.0 µm)</td>
</tr>
<tr>
<td>Type II Meronts</td>
<td>8. spindel shaped (1.0 x 2.0 µm)</td>
<td>8. spindel shaped (0.5 x 2.0 µm)</td>
<td>Many, circular shaped (1.0 x 1.0 µm)</td>
<td>Many, circular shaped (0.5 x 0.5 µm)</td>
</tr>
<tr>
<td>Type II Meronts</td>
<td>Spherical shaped, PV membrane bounded (2.0 x 2.0 µm)</td>
<td>Spherical shaped, PV membrane bounded (2.0 x 2.0 µm)</td>
<td>Rodact-like shaped (0.5 x 0.5 µm)</td>
<td>Variable shapes (average 15.0 x 10.0 µm)</td>
</tr>
<tr>
<td>Type II Meronts</td>
<td>Spherical shaped (0.5 x 0.5 µm)</td>
<td>Spherical shaped (0.5 x 0.5 µm)</td>
<td>Spindel shaped with pointed end</td>
<td>Spindel shaped</td>
</tr>
</tbody>
</table>
Table 9.2: Comparison of the *Cryptosporidium* life cycle in different environments. Data from the present study and others (Hijjawi *et al.*, 2002; Hijjawi *et al.*, 2004; Petry, 2004; Valigurová *et al.*, 2008; Petry *et al.*, 2009; Borowski *et al.*, 2010; Hijjawi *et al.*, 2010; Edwards *et al.*, 2012; Valigurová, 2012)

<table>
<thead>
<tr>
<th></th>
<th>In-vivo</th>
<th>In-vitro</th>
<th>Cell-Free</th>
<th>Biofilms</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Micronemes</strong></td>
<td>Oval shaped (6.0 x 3.3 μm)</td>
<td>Oval shaped (5.3 x 3.3 μm)</td>
<td>Oval shaped (5.0 x 5.0 μm)</td>
<td>Variable shaped (5.0-10.0 μm)</td>
</tr>
<tr>
<td><strong>Macronemes</strong></td>
<td></td>
<td>Many</td>
<td>Many</td>
<td>Many</td>
</tr>
<tr>
<td><strong>Merozoites</strong></td>
<td>Spherical shaped (4.0 x 4.0 μm)</td>
<td>Oval shaped (4.0 x 3.0 μm)</td>
<td>Oval shaped (5.0 x 5.0 μm)</td>
<td>Oval shaped (2.5 x 3.0 μm)</td>
</tr>
<tr>
<td><strong>Mesozoites</strong></td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Ontozoites</strong></td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Sporozoites</strong></td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Binary Fission</strong></td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Plasmodicity</strong></td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Gamont-like Stage</strong></td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Extra Large Gamont-like Stage</strong></td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes (&gt; 30 μm)</td>
</tr>
</tbody>
</table>
Table 9.3: The comparison of gregarines and Cryptosporidium life cycles in general. Data from present study (biofilm systems) and others (Corliss, 1962; Gunderson and Small, 1986; Bandyopadhyay *et al.*, 2004; Costa-Leonardo *et al.*, 2008).

<table>
<thead>
<tr>
<th>Multiplication process</th>
<th>Gregarines</th>
<th>Cryptosporidium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epicellular Multiplication</td>
<td>Briefly via aecuron or epimerite formation</td>
<td>Can complete its entire life cycle epicellularly in the host.</td>
</tr>
<tr>
<td>Extracellular Multiplication</td>
<td>Once mature trophozoites are formed, they unattach from the host cells and complete the rest of the life cycle extracellularly.</td>
<td>The entire life cycle can be completed extracellularly in cell-free cultures and possibly in a biofilm system. Also has the ability to multiply extracellularly in the host when epithelial cells invasion fails.</td>
</tr>
<tr>
<td>Multiplication process</td>
<td>Gametogony, sporogony, binary fission, syzygy</td>
<td>Merogony, gametogony, sporogony</td>
</tr>
<tr>
<td></td>
<td>No merogony stage</td>
<td>Cell free culture showed that Cryptosporidium have the capability to skip the merogony process.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Binary fission was only observed in <em>in-vitro</em> and biofilm system.</td>
</tr>
<tr>
<td>Syzygy</td>
<td>Head to head/side to side</td>
<td>Head to head in <em>in-vitro</em> and cell free cultures</td>
</tr>
<tr>
<td></td>
<td></td>
<td>In the biofilm systems:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&lt; 4 trophozoites side to side</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&gt; 4 trophozoites: both side to side and head to head</td>
</tr>
<tr>
<td>Developmental Stages</td>
<td>Sporozoites, trophozoites, gametocytes, gametes</td>
<td>Sporozoites, trophozoites, merozoites, merozoite, gamont-like stages (<em>in-vitro</em> and cell free culture; biofilm systems), extra large gamont like stages (biofilm systems)</td>
</tr>
<tr>
<td>Diversity of Morphology</td>
<td>Variable morphologies that are highly dependent on the nutrient availability in the surrounding environment.</td>
<td>Variable morphologies that are highly dependent on the nutrient availability in the surrounding environment.</td>
</tr>
<tr>
<td>Size Range of Developmental Stages</td>
<td>1 μm – above 50 μm.</td>
<td><em>In-vivo</em>, <em>in-vitro</em> and cell free cultures: 0.1 μm to 5 μm</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Biofilm systems: 0.1 μm to above 50 μm.</td>
</tr>
</tbody>
</table>
This PhD study shows the potential risk posed by biofilms and Cryptosporidium where treatment barriers are challenged, not working or non-existent. However, the public health significance of this in relation to waterborne outbreaks has yet to be determined. These risks will be dependent on a range of factors including: whether development can occur under the very low nutrient conditions found in drinking water biofilms and whether development in drinking water biofilms goes through to the oocyst stage.

In addition, the present study showed that biofilms influence the Cryptosporidium life cycle as evidenced by the observation of various developmental stages that have never been observed in *in-vitro*, cell-free cultures or *in-vivo*. The infectivity and their resistance to chlorine need to be further determined, as does whether these stages are carried back into water during biofilm sloughing or erosion. Furthermore, this study demonstrated that *Pseudomonas* produce biofilms that support Cryptosporidium growth in an aquatic environment. However, as different bacteria/mixed species produce different types of biofilms and microenvironments (Percival *et al.*, 2000), they may generate nutrients that are more readily assimilable or unassimilable by *Cryptosporidium*. Therefore, it is necessary to determine whether natural biofilms (mixed-species) or biofilms produced by certain bacterial species better support (or conversely more adversely affect) the survival and development of *Cryptosporidium* than others.

In conclusion, the findings generated from this thesis will increase the understanding of the potential risk posed by *Cryptosporidium* in association with biofilms and provide better distribution strategies at those times where *Cryptosporidium* may be present in distribution system. The current study shows that biofilms have the capacity to provide a niche for the accumulation of nutrients that can then be made
available to Cryptosporidium to develop and to increase in number during their retention period. As such, the control of Cryptosporidium development and biofilms within aquatic environments will need to be addressed in the future to maintain quality drinking water.

9.1 References


