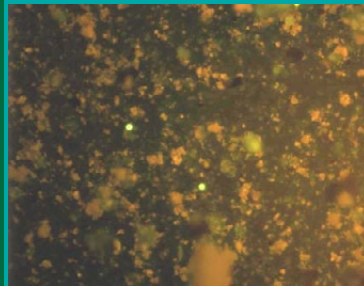
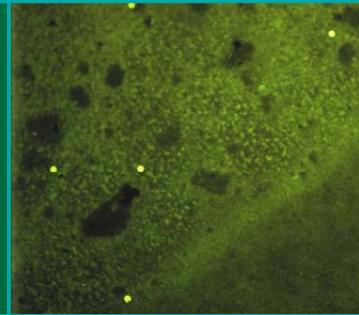


The Cooperative Research Centre for

Water Quality and Treatment



Cryptosporidium Oocyst Interactions with Drinking Water Pipe Biofilms



Research Report 5

Research Report

5

***Cryptosporidium* Oocyst Interactions with Drinking Water Pipe Biofilms**

Malcolm Warnecke

Sydney Water

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Foreword

Research Report Title: *Cryptosporidium* Oocyst Interactions with Drinking Water Pipe Biofilms

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CRC for Water Quality and Treatment Project No. 4.2.2.1 – *Cryptosporidium* oocyst and virus interactions with drinking water pipe biofilms

Executive Summary

The behaviour of the parasite *Cryptosporidium* in drinking water distribution systems is not well defined. In particular, interactions between *Cryptosporidium* oocysts and biofilms attached to pipe surfaces have not previously been specifically examined. The potential exists for oocysts to become attached to pipe biofilms, and subsequently to detach intermittently or following a change in system conditions. Disinfection of water with chlorine is unlikely to affect *Cryptosporidium* oocysts but may cause detachment of biofilm containing attached oocysts.

A pilot-scale pipe-rig was constructed to examine *Cryptosporidium*-biofilm interactions. The primary concern was to correctly simulate pipe surface conditions, so the system was predominantly composed of exhumed sections of 100mm drinking water pipe. The system was intended to simulate a slow-flowing area of water main under two chlorination conditions. Six experimental runs were performed in total. The initial half of all experiments was the same, and was performed using dechlorinated water. The second half of three of the runs were intended to simulate superchlorination conditions following a main break and repair. The second half of the remaining runs simulated conditions after chlorination of an upstream reservoir.

The experimental system was allowed to develop with dechlorinated water for a week, and was then inoculated with approximately 10^6 oocysts, inactivated by gamma-irradiation. Following a period of recirculation, dechlorinated input water was introduced. Displaced water from the pipe-rig was collected and examined for oocysts. The main break and repair simulation followed the initial conditions with a 1h period of 20mg/L chlorine, followed by emptying the system, and then again using 0mg/L chlorine input water. The simulation of chlorination of an upstream reservoir followed the initial conditions with input of 2mg/L chlorinated water.

The water and biofilm samples were examined for oocysts and a range of other experimental parameters. Oocyst samples were concentrated using membrane filtration and flow cytometry for water samples, and immunomagnetic separation for biofilm samples. Concentrates were then examined using immunofluorescence microscopy.

The majority of *Cryptosporidium* oocysts introduced into the system were not recovered in water samples from the pipe-rig. It was hypothesised that most of these oocysts were probably attached to surfaces within the system, although this was not confirmed by the direct examination of the surface biofilm. This may have been due to heterogenous distribution, inaccurate recovery estimation due to oocyst attachment and embedding, and oocyst destruction and disintegration. Oocyst recovery from water samples was regarded as accurate, with recovery typical of this type of sample. Results from water samples did not provide information on numbers of surface-attached oocysts.

Contaminated pipe surfaces were shown to be capable of intermittent release of oocysts over an extended period of time. Direct observation of resuspended biofilm showed some attached oocysts.

A theoretical model of the experimental system as a chemostat with no surface interaction predicted substantially different oocyst outputs to those observed experimentally. The difference between theoretical and observed behaviour could be explained by the majority of oocysts initially attaching to surfaces, with subsequent intermittent detachment.

High dose chlorination (20mg/L) for 1h resulted in substantial release of oocysts from a contaminated pipe surface, although the majority of oocysts remained attached to the surface. Continuous dose chlorination at 2mg/L in this system resulted in almost complete reduction of chlorine in the water phase. This level of chlorination did not result in large-scale resuspension or detachment of oocysts from the pipe biofilm, which may have resulted from biofilm modification caused by chlorine oxidation.

Based on the surface attachment and subsequent intermittent detachment of oocysts observed in the pipe-rig, it is expected that variability between grab samples over time in an actual distribution system would be high. In this case, composite samples taken over time or space would provide better estimates of oocyst concentrations compared to grab samples. Nevertheless based on the observed variability of oocyst detection, the setting of arbitrary acceptable levels of oocysts in drinking water should be discouraged. Heterogeneous distribution and uncertainty with oocyst recovery from other material, also makes ad-hoc biofilm or sediment samples of limited value.

These findings support a risk-based approach to ensuring drinking water quality. In a risk-based approach, emphasis is placed on improved catchment management practices to limit or at least understand the inputs of *Cryptosporidium* into source waters, and treatment optimisation to remove oocyst before they enter distribution systems. End point sampling, such as in a distribution system is not recommended as an effective means of predicting or managing risk.

The interaction of shear stress and oocyst detachment and resuspension remains incompletely understood. How long attached oocysts remain viable and infective, and once non-viable how long they can be detected is also unclear. Desirable technical advances would include improved isolation of *Cryptosporidium* from surface materials such as biofilm or sediments. Further experimentation and development towards these objectives is required to better understand *Cryptosporidium* behaviour in drinking water distribution systems, and consequently develop informed managerial strategies to deal with distribution system compromise and the risks associated with it.

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1. Introduction

1.1 Aim

The aim of this project was primarily to examine interactions between *Cryptosporidium* oocysts and biofilms attached to drinking water pipe surfaces. This is limited to examining evidence of transfer between the bulk water phase and elsewhere, while not closely examining transfer mechanisms.

This introduction summarises some of the recent literature on the behaviour of *Cryptosporidium* as a waterborne pathogen, of biofilms in drinking water systems, interaction between *Cryptosporidium* and water system biofilms, and the relevant effects of chlorine.

1.2 *Cryptosporidium* in drinking water

In recent years, the protozoan parasite *Cryptosporidium parvum* has become the largest single health issue in the drinking water industry in the developed world. This has been largely due to its isolation as the etiologic agent in several large waterborne outbreaks, and growing numbers of more susceptible (immunocompromised) people within the community. There has been explosive growth in scientific study of this area in the last decade, although many questions regarding the behaviour of *Cryptosporidium* remain unanswered.

Biology of *Cryptosporidium*

Cryptosporidium infections are confined to an intracellular, extracytoplasmic location. This is usually located at the microvillous surface of an intestinal host cell. The infection involves several stages of the *Cryptosporidium* life cycle. This includes a thin-walled autoinfective oocyst stage, thought to cause further infection within the same host. Also produced is a thick-walled, environmentally resistant oocyst, which is egested with the faeces of the host, and is responsible for transmission of infection to a new host (Garcia *et al.*, 1993). The oocyst itself is round, approximately 4-6µm in diameter, contains 1 to 4 sporozoites, and may show other characteristic morphological features.

Oocyst transmission can be via various faecal-oral routes, including drinking water containing faecal contamination. An endemic level of infection within the community can be expected. Generally, a patient with a normal immune system will have a self-limited infection with gastroenteritis symptoms. However, an immunocompromised patient may have a chronic infection, with symptoms ranging from asymptomatic to severe (Garcia *et al.*, 1993).

Other than in the immunocompromised population, symptomatic infections are generally mild. This can make identification of an outbreak somewhat difficult, as outbreak cases are submerged in the

background levels of gastroenteritis cases in the overall population. For example, it has been estimated that an outbreak of 1,000 cases in New York City could easily be overlooked within the usual background incidence of diarrhoea (Craun *et al.*, 1998; and references within).

Detection methods

Cryptosporidium parvum is generally accepted as having a low infectious dose; a dose-response study by DuPont *et al.* (1995) found a median infective dose of 132 oocysts for the strain examined. There would appear to be considerable variation in infective dose between strains (see Casemore, 1990; Meinhardt *et al.*, 1996; Chappel *et al.*, 1997), with strains most infective in humans likely to have the smallest infective dose.

Consequently, large volumes of drinking water are generally examined, typically in the range of 10 to 100L. Methods enabling concentration and separation of these volumes have improved in recent years, contrasting with poor and variable recoveries when using techniques initially proposed for this purpose (Badenoch, 1990; ASTM, 1992; Clancy *et al.*, 1994; Nieminski *et al.*, 1995). All current methods involve an initial step of reduction of sample volume to 1L or less, this is usually accomplished using a filtration process followed by an elution step.

Antibody-based separation methods have become increasingly useful in recent years, and are incorporated in the latest attempt to standardise detection techniques (USEPA, 2005). Included in these antibody-based methods is the application of flow cytometry and immunomagnetic separation (IMS). Flow cytometry involves analysis of a sample concentrate stained with a fluorescent-labelled antibody. The sample is processed in a flow stream illuminated by a light source, droplets within the flow stream emitting an appropriate fluorescent signal are then sorted to a membrane filter or glass slide for subsequent microscopic examination. Originally utilised for clinical requirements, this technique has been developed for application to environmental water samples (Vesey *et al.*, 1993; Vesey *et al.*, 1994; Hoffman *et al.*, 1997). More commonly used in routine laboratories, however, IMS involves mixing a sample concentrate with magnetic beads conjugated to an antibody, thereby attaching the beads specifically to the target oocysts. A magnet is used to selectively concentrate the conjugated oocysts, the beads are then dissociated, and the concentrate further handled using flow cytometry and/or microscopic examination. The application of this technique is described elsewhere (Bukhari *et al.*, 1998; Reynolds *et al.*, 1999, USEPA, 2005). Specificity of these separation techniques and the subsequent specificity of fluorescent staining is dependent upon the antibody preparation used; such specificity can vary significantly between commercially available antibodies (Ferrari *et al.*, 1999).

Drinking water outbreaks and endemic disease

The resistance of oocysts to disinfection by chlorine (Korich *et al.*, 1990; Finch *et al.*, 1994; Venczel *et al.*, 1997; Liyanage *et al.*, 1997; Finch *et al.*, 1997) emphasises the need for water utilities to use removal processes such as filtration, and to protect source waters from faecal contamination. This conclusion supports the current risk-based approach to ensuring water quality, as advocated by

drinking water guidelines, including those in Australia (WHO, 2004; NHMRC, 2004). This approach is established on the use of multiple barriers, such as catchment management and water treatment, to prevent microorganisms, in this case *Cryptosporidium*, from entering drinking water supplies. Nevertheless, on the rare occasions when these processes do not perform optimally then *Cryptosporidium* may enter a distribution system with the chance of outbreaks and endemic disease occurring.

Reported waterborne outbreaks of cryptosporidiosis have increased in recent years, due to awareness of the problem, and to improvements in detection techniques. Indeed, the most frequently identified waterborne etiologic agents in outbreaks of gastroenteritis in the United States in the past 12 years have been *C. parvum* and *Giardia lamblia* (Craun *et al.*, 2006). Nevertheless, the probability of acquiring cryptosporidiosis sporadically from consuming drinking water is low (Hughes *et al.*, 2004; Hunter *et al.*, 2004; Khalakdina *et al.*, 2003; Robertson *et al.*, 2002; Roy *et al.*, 2004), but is dependant on the quality of source water and level of treatment (Goh *et al.*, 2004).

Distribution systems

A review of United States waterborne disease outbreaks (where two or more persons experience a similar illness) covering all aetiological agents not just *Cryptosporidium*, showed that outbreaks associated with untreated surface water sources has decreased since 1971, with no waterborne disease outbreaks reported for untreated surface waters since 1991 (Craun *et al.*, 2006). This is largely due to efforts by the USEPA to regulate for adequate treatment of surface waters. Furthermore, while treatment deficiencies have also become less important as a cause of waterborne disease outbreaks, they still accounted for 14% of waterborne disease outbreaks from 2001 to 2002 (Craun *et al.*, 2006). Typically in such outbreaks, an unusual event caused increased contamination of the water source, and a specific deficiency or deficiencies were identified in the treatment process. Under such conditions it is likely that distribution systems will become contaminated.

Surprisingly, therefore, the fate of oocysts once in a distribution system remains largely unknown. This has important implications as disinfectant residuals that are able to inactivate bacterial pathogens are ineffective against *Cryptosporidium*. Distribution systems are complex heterogeneous environments that while serving as a conduit, also have the potential to interact with particles passing through it. This is particularly the case at substratum-water interfaces, as described below.

1.3 Biofilms in drinking water distribution systems

Background

A water distribution system, containing low levels of nutrients and often biocides at levels designed to kill microorganisms, would not seem to be a good microbial growth environment. However, the universal presence of heterotrophic bacteria within these systems demonstrates the hosting of microbiological activity.

Bacterial interaction with surfaces in these systems is one of the more important growth determinants. In pilot-plant studies it has been shown that growth in the bulk liquid phase is negligible, and that planktonic increases are due primarily to detachment of biofilm cells and “breakthrough” of cells through water treatment plant barriers (Van der Wende *et al.*, 1989; Bucklin *et al.*, 1991; Block, 1992).

A biofilm has been defined as a surface accumulation of cells immobilised at a substratum, frequently embedded in an organic polymer matrix of microbial origin, which may contain a significant fraction of inorganic or abiotic substances (Characklis and Marshall, 1990). Attachment can provide a favourable growth environment for bacteria. As summarised by Fletcher and Marshall (1982),

- substrata-attached cells do not have to waste energy searching for food, as water containing fresh nutrients flows over them and removes waste products;
- they are prevented from washing through the system;
- the substratum to which cells are attached may also be a growth substrate; and
- nutrients tend to absorb to surfaces and are thus more available to surface attached cells.

It is now generally accepted that biofilms on distribution pipes may occasionally cause, or contribute to drinking water quality degradation in distribution systems and hence challenge a water authority’s ability to deliver high quality water. This is primarily through

- the incorporation and subsequent release of potential pathogens, under appropriate conditions
- impacting on disinfectant decay
- contributing to the nitrification of chloramines
- facilitating the corrosion of iron pipes

The protective effect of bacterial attachment from disinfection is also very significant. Several studies have found attachment by bacteria significantly enhanced resistance to disinfection in water systems (LeChevallier *et al.*, 1984 and 1988; Herson *et al.*, 1987).

A heterogenous distribution of biofilm can be expected through a distribution system. Parameters such as shear stress, disinfectant residual, residence time and nutrient levels will determine the amount of biofilm development in any given area.

Biofilm development can be expected on sediments, with inorganic particles serving as a substratum. Sediments can serve as a protected reservoir for attached microorganisms, and as a vehicle of transmission should they become resuspended.

The direct effects of microbial growth in the distribution system on water quality and public health are unequivocal. A series of epidemiological studies undertaken in Canada investigating low-level gastroenteritis due to drinking water (Payment *et al.*, 1991; Payment *et al.*, 1993) associated a large

proportion of symptoms with water consumption. It appeared that residence time in the distribution system was associated with symptoms, implying a contribution from bacterial growth. In contrast, a subsequent study on the same area (Payment *et al.*, 1997) found a lack of association of gastroenteritis with residence time in the distribution system. In this second study, it was noted that water residence time never exceeded 34 hours, and that localisation of symptoms may have been due to contamination events within the distribution system. Evidence to date suggests a lack of correlation between drinking water consumption and illness in major Australian cities. A case controlled epidemiological study in Melbourne found no connection between the consumption of drinking water and gastrointestinal illness (Hellard *et al.*, 2001). The Melbourne drinking water supply, while not filtered, is chlorinated and the source water is obtained from very well protected catchments. Similarly, a study of sporadic cryptosporidiosis in Melbourne and Adelaide, two Australian cities with different source water characteristics and levels of treatment, found no correlation between consuming drinking water and illness (Robertson *et al.*, 2002).

In summary, bacterial biofilms can be expected to develop, with a heterogenous distribution, throughout a drinking water distribution system. The majority of microbial activity within the system can be expected in the biofilm, resulting in degradation of water quality. Some interaction can be expected between the biofilm and other organisms being transported through the system.

Biofilm reactors

Investigations of biofilm properties in water systems cannot always be performed in actual water systems. This is due to difficulties with accessing biofilms on pipe surfaces, the inability to introduce pathogens into distribution systems to study their fate, and not having sufficient control over water quality parameters. It is frequently more appropriate to perform experimental studies in pilot or laboratory scale reactors under controlled conditions.

The choice of reactor is largely based upon the requirements and goals of the experiment, and the flow characteristics of the system to be simulated. This topic has been extensively discussed by Characklis (1990a).

1.4 *Cryptosporidium*-biofilm interactions

Previous studies

There has been little work directly investigating *Cryptosporidium* interacting with surfaces in a drinking water distribution system environment. One study investigated oocyst behaviour in a small laboratory-scale chemostat system, using glass slides to develop biofilms (Rogers *et al.*, 1995). Attachment was noted as occurring with the majority of introduced oocysts. This attachment was observed occurring in clusters, and the possibility raised of sloughing releasing small but effectively infectious quantities of oocysts. This study raised several important questions, although its findings cannot be applied directly

to a water distribution system environment due to assumptions and simplifications necessary for the study.

This study also showed extended viability of attached oocysts within the test system, with a substantial proportion remaining viable after 35 days of attachment (Rogers *et al.*, 1996).

There have been some examinations of the surface properties of *Cryptosporidium* oocysts. Brush *et al.* (1998) demonstrated that the method of purification of oocysts altered their adhesion properties, and that hydrophobicity can change as oocysts age. These changes would affect any study involving surface interactions. A study examining sedimentation rates of oocysts mixed with settled secondary treated sewage effluent (Medema *et al.*, 1998) found the majority of oocysts attached to particles following mixing. Sedimentation rates were then determined by the properties of the particles. When applied to aquatic systems this implies an accumulation of oocysts in sediments.

Studies examining particle interaction with biofilms are also of relevance here. A biofilm interaction study by Drury *et al.* (1993), using 1µm latex beads of specific gravity 1.05 and negative surface charge, observed an accumulation and an extended retention period for the beads towards the biofilm substratum. It was hypothesised that surface roughness was a mechanism for particle transport within the biofilm. A study examining the interaction of 1.2-50µm carbon fines with biofilms (Camper *et al.*, 1995; Morin *et al.* 1997) found the smaller fines in this range initially attached, and were subsequently eliminated from the biofilm. The larger fines also attached but were retained after 24 hours. When subjected to disinfection, fines larger than 5µm were lost from the biofilm, indicating a preferential loss of the carbon fines over bacterial biomass.

1.5 Effects of chlorine

Disinfection and detection of *Cryptosporidium*

Cryptosporidium oocysts are extremely resistant to chlorination. Korich *et al.* (1990) found that a 90 minute exposure to 80mg/L of chlorine at pH 7 resulted in a 90% inactivation of oocysts. Subsequent research has shown greater effect from chlorine (Finch *et al.*, 1994; Venczel *et al.*, 1997; Liyanage *et al.*, 1997; Finch *et al.*, 1997). A concentration of 1-2mg/L of chlorine, as normally used in drinking water disinfection, cannot be expected to inactivate oocysts.

Also of relevance is the effect of chlorine upon oocyst detection by immunofluorescent antibody (IFA) techniques. Sufficient exposure to chlorine results in the prevention of antibody binding to the oocyst wall, presumably due to oxidation of external epitopes. Previous internal method development work (unpublished) has shown that exposure of greater than 20mg/L for 20h was required prior to loss of detection. Environmentally aged oocysts may also be degraded, and more vulnerable to chlorine oxidation.

Interactions with biofilms

Chlorine can be consumed in side-reactions, referred to as chlorine demand. Chlorine demand in water systems can be due to suspended organics and other materials as well as reactions with biofilms and surfaces within the system.

Chlorine acts against biofilms by both killing microorganisms, and oxidation of extracellular polysaccharides (EPS) resulting in biofilm depolymerisation, dissolution and detachment (Characklis *et al.*, 1980). The reaction of chlorine is extremely rapid. With regards to disinfection capacity, chlorine has been found to be most effective at pH 6 to 6.5, where hypochlorous acid dominates other chlorine species. The pH of water in distribution systems is typically higher, with an increased proportion of hypochlorite ion amongst chlorine species. Pilot biofilm destruction studies have found improved action at higher pH levels, suggesting that the ionic form of chlorine may be more effective at depolymerisation of EPS (Characklis, 1990b).

A reactive disinfectant such as chlorine in the bulk phase can be expected to interact with the areas of a pipe biofilm closest to the water interface (de Beer *et al.*, 1994; Huang *et al.*, 1995). If oocysts had previously interacted with the biofilm, they may have attached to these outermost areas. If this was the case, subsequent disinfection would depolymerise the areas containing oocysts, resulting in their possible resuspension. However, should the oocysts penetrate the biofilm and be retained close to the substrata, as previously observed with latex microbead studies (Drury *et al.*, 1993), then chlorination would cause a relatively small amount of oocyst resuspension.

2. Materials and Methods

2.1 Pipe rig

The experimental system used contained two sections of 100mm diameter exhumed drinking water pipe. These sections were cement-lined ductile cast iron, with an internal diameter of 90mm. Pipe condition was relatively good, age varied between sections, but was estimated as about seventy years in-service for one section. Each of these sections was approximately 1m in length, and was replaced for each experimental run. The pipe sections were connected by a 180° elbow joint of new pipe at one end, and peristaltic tubing attached to end caps at the other end. The total volume of the rig ranged between 20L and 22L, depending upon the exact lengths of pipe.

Joints between pipes were closed using cable ties and hose clamps, and sealed using a silicone sealant (Selleys Roof and Gutter). Water circulation was provided by a peristaltic pump on the tubing, giving a flow velocity in the pipe of 0.01m s^{-1} . In addition, Sydney tap water was pumped into the system using a second pump, following alteration of chlorine concentration, at a rate to give a mean residence time of 20h within the rig. Chlorine alteration was performed in batch by addition of bleach (Orica Chemicals, liquid swimming pool bleach) or sodium thiosulfate (APS Chemicals, laboratory reagent grade) to a 60L drum of tap water, with chlorine level determined (DPD method) following mixing and equilibration. Water displaced from the system was collected in 20L or 60L sampling drums, and was examined as the experimental samples were. A diagram of the pipe rig is shown as Figure 1. Biofilm samples of 25cm^2 , sampling a 5cm by 5cm area, were taken at certain times during the experiments, from points A and B (Figure 1). The initial biofilm sample taken from a point was always the lowest section of the pipe, subsequent samples were from the surrounding area.

2.2 Experimental design

The pipe-rig system was intended to simulate a slow-flowing area of main under two chlorination conditions. Six experimental runs were performed in total. The initial half of all experimental runs was the same, and was performed using dechlorinated water. The second half of three runs were intended to simulate conditions following a main break and repair. The second half of the remaining runs (3) simulated conditions after chlorination of an upstream reservoir.

The pipe-rig system was run for seven days prior to oocyst inoculation, to allow existing biofilm to adapt to the experimental conditions. Input water was dechlorinated using sodium thiosulfate prior to introduction to the system. A 20L sample of output water was collected and seeded as a positive control for recovery purposes.

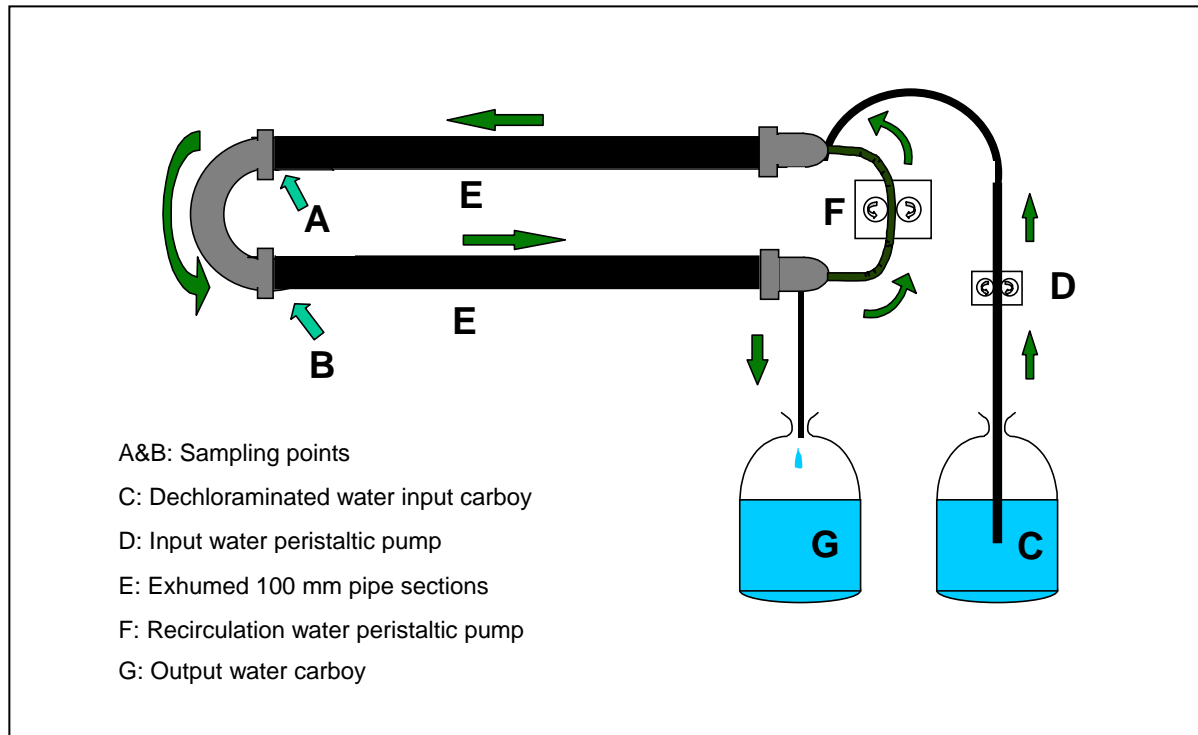


Figure 1: Pipe loop diagram

An inoculum of oocysts was introduced to the system at time -20h . Following inoculation, water in the pipe system was circulated, but there was no input or output with the system for 20h . At time 0h , input and output commenced. Carboys, providing input and collecting output (C and G Figure 1), were changed according to the experimental schedule, which generated the following samples:

Initial conditions

Output water was collected as samples during the following time periods of the initial phase of the experiment in all six runs.

0h to 20h

20h to 40h

40h to 80h

80h to 120h

120h to 160h

160h bulk

160h biofilm

As noted, the pipe loop was emptied at 160h to provide the “bulk” sample, and then opened to provide the “biofilm” sample at point A. The system was then closed and resealed. Further actions were dependent upon which simulation was performed.

Mains break simulation (Runs 1, 2 and 3)

Output water was collected as samples during the following time periods of the subsequent phase of the experiment in runs 1, 2, and 3.

165h bulk

165h biofilm

168h to 188h

188h to 208h

208h to 248h

248h to 288h

288h to 328h

328h bulk

328h biofilm

The rig was resealed after the 160h sample, with 2h taken for adequate setting of sealant. The system was then filled with 20mg/L free chlorine, and left for 1h. The last 5min of this included circulation flow within the system. The pipe was then pumped out, with this water dechlorinated following chlorine level determination, and composing the 165h bulk sample. The system was reopened, and another biofilm sample taken at point A. The system was resealed, with 2h taken for adequate setting of sealant. Further input was with dechlorinated water as before, with periodic output samples taken for another 160h. At 328h, the pipe was again drained and opened, providing a bulk water sample. Biofilm samples were also taken at points A and B, as shown on Figure 1.

Upstream chlorination simulation (Runs 4, 5 and 6)

Output water was collected as samples during the following time periods of the subsequent phase of the experiment in runs 4, 5, and 6.

168h to 188h

188h to 208h

208h to 248h

248h to 288h

288h to 328h

328h bulk

328h biofilm

The rig was resealed after the 160h sample, with 7h taken for setting of sealant. The system was then filled with 2mg/L free chlorine, which was taken through chlorine breakpoint during preparation. Recirculation, input and output were restarted and continued for 160h. Further input was with 2mg/L free chlorine. At 328h, the pipe was again drained and opened, providing a bulk water sample. Biofilm samples were also taken at points A and B, as shown on Figure 1.

2.3 Oocyst inoculum

Faecal material was collected in a sterile sample container, following direct stimulation of diarrhoeic pre-weaned (7 to 21 day old) calves from a large local commercial dairy farm. This material was then transported to the laboratory.

The faecal material was initially desegregated by filtering through several layers of cotton gauze, using ice cold distilled water as a wash solution. Centrifugation followed by pellet resuspension in ice cold distilled water was performed three times. The desegregated material was then pipetted to the surface of a layered Percoll 1.05/1.09 SG gradient tube, following centrifugation the material at the density interface was harvested, and resuspended in ice cold distilled water. This material was washed by centrifugation and resuspended in ice cold distilled water three times. The final pellet was resuspended in 1.5mL distilled water and transferred to a microcentrifuge tube.

Due to safety reasons, the oocyst suspension used in the experiments was inactivated by gamma irradiation using 1500 Ci (5.55×10^{13} Bq) for 24h from a ^{60}Co source (Macquarie University, Sydney). Following irradiation, the oocyst stock preparation was stored at 4-8°C. This single preparation was used in all of the experimental runs and for all seeding recovery testing. The preparation was stored for several months before the initiation of the experimental runs.

Prior to inoculation of the experimental system, the stock preparation was vortex mixed, and a dilution series prepared. This was followed by staining the dilution aliquot with FITC-labelled anti-*Cryptosporidium* IgG Cry104 (PanBio, Brisbane), and enumeration by fluorescence microscopy. Counting techniques and criteria for oocyst identification are described below in "Microscopy" (Section 2.7).

The inactivated oocysts inoculated to the system were not pre-labelled with antibody. This may have simplified subsequent detection, but may also have altered the surface characteristics of the oocysts.

2.4 Sample equipment preparation

Some carboys (60L size, Reflex Plasticware) were reused between experiments. These carboys were scrubbed with detergent (Pyroneg), followed by 12.5% bleach (Orica Chemicals, liquid swimming pool bleach), and rinsed with 5% sodium thiosulfate (APS Chemicals, laboratory reagent grade) and deionised water. Smaller samples were taken using new 25L carboys (Van Leer Australia), which

were not reused. Craft knife blades used for biofilm sampling were autoclaved prior to use. The 180° pipe bend and end caps were reused between experiments; these were scrubbed with detergent, soaked in 50mg/L bleach for 24h, and then rinsed with 5% thiosulfate and deionised water.

2.5 Sampling procedures

Water samples were generated by displacement, caused by pumping input water into the pipe-rig. These were collected in 25L (Van Leer Australia) or 60L (Reflex Plasticware) plastic carboys, for 20h or 40h sampling periods, respectively. "Bulk" samples refer to the water left in the pipe, which was emptied out at particular times. It was intended to minimise shear stresses from sampling. Bulk samples were, therefore, generated by pumping air through the recirculation hose and collecting the discharge water from the inlet attachment. Biofilm samples were taken by opening the pipe-rig at points A and/or B (Figure 1), and scraping the exhumed pipe surface with a craft knife blade. A single 5cm x 5cm area was scraped per sample, this surface material was transferred to a 50mL centrifuge tube, and volume made up to 10mL with sample buffer.

During the biofilm sampling procedure, a residual water sample containing a substantial quantity of sloughed biofilm and resuspended material was also usually generated. Such material was decanted from the water sample and transferred to a 50mL centrifuge tube. This constituted a separate sample, which was processed using IMS and flow cytometry in the same way as the biofilm samples.

2.6 Supplementary testing

Temperature and pH determination

Temperature was measured at the time of sampling (Dobros brand mercury thermometer, range -5°C to 50°C). pH was also measured at this time using a pH meter (Hanna Instruments, Model 8521-N).

Chlorine determination

Total chlorine measurements were performed at time of sampling (Hach DR100 colorimeter, with reagents for DPD chlorine determination). Measurements were also performed during preparation of input waters.

Heterotrophic plate counts

R2A (Difco) plates were used for determination of heterotrophs. Serial dilutions of sample were pipetted to R2A plates, and spread using an ethanol-flamed glass spreader. Plates were incubated at room temperature for 7 days, and resultant colonies counted.

2.7 Oocyst concentration and enumeration

Membrane concentration

Water samples were concentrated through a 293mm diameter flat-bed filter unit (Millipore), containing a 2µm pore size track etched polycarbonate membrane (Osmonics, Massachusetts, USA) using a water pump (Procon CO 1604X self priming vane type). The inside of the sample carboy was squeegeed and rinsed with 0.1% Tween 80, with the resultant washings also concentrated through the same membrane. Following concentration, the membrane was transferred to a perspex sheet. The sample concentrate was then eluted using a 0.1% Tween 80 (ICN Biomedicals, Inc) solution and a rubber squeegee, and collected in a 50mL centrifuge tube. The flat bed apparatus was cleaned between samples using 12.5% bleach, followed by rinsing with 5% sodium thiosulfate and deionised water.

The concentrate was centrifuged and washed 3 times using sample buffer (see below), transferred to a 15mL centrifuge tube, and centrifuged and washed again. The sample pellet was then forced through a 38µm stainless steel mesh (Metal Mesh Pty Ltd), and further washed with sample buffer. The resultant concentrate was then processed using flow cytometry.

Sample buffer was prepared using the following reagents per litre of deionised water: 5g bovine serum albumin (Sigma), 0.5mL Tween80, 0.53g tetrasodium pyrophosphate (Sigma), 0.5g sodium azide (Aldrich). The pH was adjusted to 8, and the buffer was filtered through a 0.2µm filter.

Immunomagnetic separation (IMS)

Scraped and resuspended biofilm samples were initially separated using IMS. Water samples with a large particulate load were also separated by IMS prior to processing by flow cytometry.

Scraped biofilm samples were resuspended to a final volume of 10mL in sample buffer. Resuspended biofilm samples of 50mL volume were taken, these were centrifuged and the supernatant aspirated to waste to give a concentrated volume of 10mL. Of the 10mL sample volume, 2.5mL was analysed by IMS. As oocyst recovery using this method on biofilm samples was unknown, an additional 2.5mL of sample was spiked with a known concentration of oocysts and processed simultaneously. Recovery was therefore calculated individually for each sample for IMS, contrasting its method of calculation with that for samples processed using flow cytometry.

Immunomagnetic separation was performed using commercially available kits (Dynal), with the procedure used derived from the manufacturer's instructions. Briefly, a quantity of sample was mixed in a flat-sided test tube with a proprietary buffer and paramagnetic beads labelled with an anti-*Cryptosporidium* antibody. The samples were affixed to a rotating mixer and rotated for at least 1h at room temperature. The tubes were removed and placed in a magnetic particle concentrator (MPC-1) and rocked by hand for 1-2min, followed by decanting of non-magnetised material. The magnet was removed, and magnetised material gently resuspended in proprietary buffer and transferred to a 1.5mL microcentrifuge tube. This was placed in another magnetic particle concentrator (MPC-M),

rocked by hand for 1min, and non-magnetic material decanted. The magnetic material was then treated with 0.1M HCl to dissociate the magnetic beads, vortex mixed, allowed to stand for 10min, and vortex mixed again.

The concentrated sample was immediately transferred to a 13mm polycarbonate membrane filter, of 0.8µm pore size (Nucleopore). The membrane was then stained with a monoclonal antibody solution (see below), mounted on a microscope slide, and examined by microscopy.

As noted, each IMS sample was run in parallel with a spiked control sample. Results were modified for parasite recovery from that sample. Recovery was calculated as follows:

$$\frac{(\text{Oocyst count in spiked recovery control sample}) - (\text{oocyst count in sample})}{(\text{Oocyst count in spike})}$$

Oocyst concentrations in the sample were then modified as follows:

$$\text{Modified count} = (\text{Oocyst count}) / (\text{Recovery})$$

Flow cytometry

The sample pellet was labelled with Cry104, an anti-*Cryptosporidium* oocyst monoclonal antibody conjugated to fluorescein isothiocyanate (PanBio), and incubated at room temperature for 15min. The sample was then processed using a FACSCalibur flow cytometer modified to process environmental water samples (Becton Dickenson Immunocytometry Systems, using CellQuest 3.1 software). Particles meeting fluorescence criteria were sorted to a 13mm polycarbonate membrane filter, of 0.8µm pore size (Nucleopore). Following processing of the entire pellet, the membrane was further stained with the monoclonal antibody solution, mounted on a microscope slide, and examined by microscopy.

Microscopy

Sample slides were examined under 200x and 400x power, using a fluorescence microscope (Zeiss Axioskop). The membrane surface was scanned, and the number of *Cryptosporidium* oocysts counted. The identification criteria used were apple-green fluorescence observed on ~5µm round objects, with staining predominantly on the edge of the object. Particles of this description with other features frequently observed on *Cryptosporidium* oocysts were also counted. These features included suture lines, and shape alterations caused by excystation along the suture line. Positive control slides containing known stained oocysts were used during counting for comparison purposes.

Recovery calculation in water samples

During each experimental run, the 20L water sample taken immediately prior to *Cryptosporidium* inoculation was used as a recovery control. This recovery control sample was spiked with a known quantity of 200-500 oocysts of the same stock used for inoculation of the test system. The recovery

control sample was mixed and allowed to settle at room temperature for 24h. The sample was then processed in the same fashion as that described for experimental samples.

Recovery data for water samples was averaged between the six experimental runs to account for variation introduced in processing. Not all samples from any run were all processed in the same batch, and therefore a range of variation could be expected. Experimental data was modified for recovery as follows:

$$\text{Modified count} = (\text{Oocyst count}) / (\text{Recovery})$$

2.8 Data handling

As noted, sample counts were corrected for recovery efficiency. As some samples were taken over longer periods of time than others, oocyst counts were expressed in output per residence times of the experimental system (20h period). Counts were then \log_{10} transformed, and comparisons performed on these modified values.

The geometric means (geomeans) of sample replicates were compared during data analysis. This was done to normalise the observed skewed distribution, due to oocyst counts of some experimental replicates being substantially larger than others.

Comparison of matched-pairs of data was performed using the Wilcoxon signed-rank test. This was considered to be more appropriate than the paired t-test, as it is suitable for a relatively small number of experimental observations, and for when the distribution of differences between observations is not necessarily normal (Helsel and Hirsch, 1992).

3. Results

3.1 Experimental design

Data was gathered over 6 experimental runs. Each experimental run was inoculated only once with inactivated oocysts. As described in the materials and methods, following inoculation the initial data (0h to 160h) was the same in all experiments, with an unchlorinated system and total system output collected in multiples of the system residence time (20h). At this point the pipe-rig was drained (forming a “160h bulk” sample) and opened, with samples taken of surface material of the pipe, and in some cases of resuspended material.

In the first three runs, following the “160h bulk” sample, the system was refilled with 20mg/L chlorinated water, which was left for an hour and then drained (forming the “165h bulk” sample), and again opened for sampling of pipe surface material. The system was then refilled with 0mg/L chlorinated water, and fed with unchlorinated water in a similar fashion to the first part of the experiment (168h to 328h). At this stage the pipe was drained (forming the “328h bulk” sample) and opened, with samples taken of surface material of the pipe, and in some cases of resuspended material.

The final three runs, following the “160h bulk” sample, the system was refilled with 2mg/L chlorinated water, and fed with 2mg/L chlorinated water in a similar fashion to the first part of the experiment for an additional 160h (168h to 328h). At this stage the pipe was drained (forming the “328h bulk” sample) and opened, with samples taken of surface material of the pipe, and in some cases of resuspended material.

Oocyst data generated has been adjusted for estimated recovery and normalised for volume or surface area. This data is presented in tabular form in Appendix 1. Data from supplementary tests (temperature, pH, HPCs, chlorine) has been normalised for volume or surface area where appropriate, and is presented in Appendix 2.

3.2 *Cryptosporidium* oocyst data

Microscopic appearance

The reading of slides generated from water samples was quite straightforward. Water input to the system was high-quality tapwater, and relatively free of fluorescent particles which can be mistaken for *Cryptosporidium* oocysts. Water output from the system was usually turbid, probably due to microbial growth and release of corrosion products into the water phase. However, it did not present problems with membrane concentration, and was also relatively free of *Cryptosporidium*-like

fluorescent particles. The FITC-labelled anti- *Cryptosporidium* IgG Cry104 did not non-specifically stain background material, which may be a significant problem with other commercially available, less specific antibodies (Ferrari, 1999). Consequently, observed oocysts were well stained against a non-fluorescent background, avoiding problems sometimes encountered with environmental samples.

A representative microscopic view typical of a water sample slide is shown in Figure 2. As can be seen, there is little or no extraneous material present, which allowed rapid and accurate reading of these slides. There are 5 oocysts present on this image, they are the bright, apple-green circles of ~5µm diameter. This and the other images presented were acquired under 400x magnification. The oocyst at the bottom of the image has excysted, this is evident from the characteristic appearance of a missing segment from the circle.

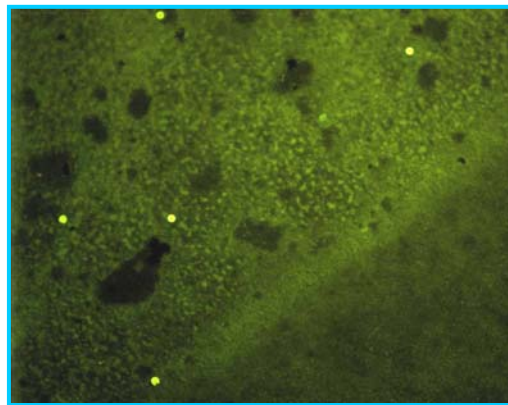


Figure 2: Representative image, water sample

The “bulk” samples contained a larger amount of suspended material, although this was predominantly removed by the flow cytometry process. Some of this material is faintly visible in Figure 3, but it did not interfere with slide reading. There are 5 oocysts present in this image, there are other faintly fluorescent particles also present which do not exhibit *Cryptosporidium*-like morphology. Note the two attached oocysts in the centre-right of the image.

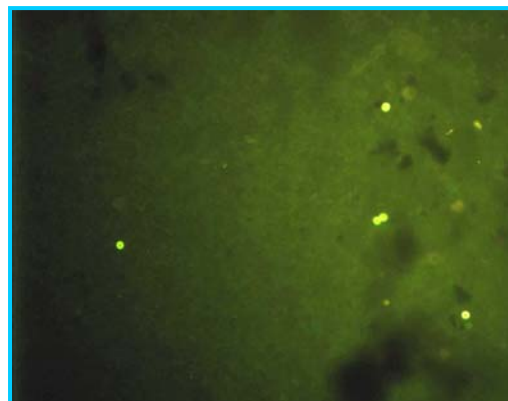
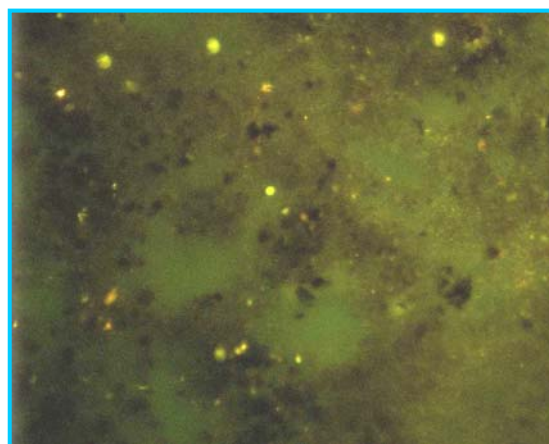


Figure 3: Representative image, bulk sample

Biofilm samples were separated using IMS, as these contained larger amounts of extraneous material. As can be seen in Figure 4, this material was not similar to the oocysts, and did not obscure the viewing area. This image has 3 oocysts present, a large amount of other reddish material, and some other areas of faintly green fluorescent material.

**Figure 4: Representative image, processed biofilm sample**

The sloughed biofilm material, mounted and stained intact, showed little background fluorescence, oocysts when seen were monodispersed and not clumped. A clumped distribution may be possible, although none was observed in these experiments. An example is shown in Figure 5. As with the other sample types, background fluorescence does not hamper examination of the slide. There is one oocyst present in the centre of this image, the other circular areas of greenish fluorescence do not have the morphology of *Cryptosporidium* oocysts.

**Figure 5: Representative image, sloughed biofilm sample**

To further examine the spatial distribution of surface-attached oocysts, some intact sections of internal pipe surface material were removed from the experimental system, stained with anti-*Cryptosporidium* antibody, and examined microscopically. These samples demonstrated substantial background fluorescence, an uneven surface, and loss of structure during the staining process (results not shown). It was decided, therefore, to persist with the examination of sloughed material to determine oocyst spatial distribution on surfaces.

Inoculum and recovery data

The oocyst inoculum level was calculated by dilution series, antibody staining and microscopy before each inoculation. The average inoculation consisted of 9.26×10^5 inactivated oocysts, with a standard error of 0.3×10^5 ; there was little variation in the stock solution between experimental runs. This preparation procedure is direct and does not involve recovery losses as described in the preparation of experimental samples. Oocysts from the stock preparation were monodispersed when observed, and did not exhibit clumping.

A sample taken prior to inoculation of each run was seeded with oocysts, processed and examined for recovery purposes. The average recovery was calculated as 41%, with a standard error of 4% (n=6, standard deviation 11%). This average recovery was applied to all water samples to calculate oocyst numbers present.

Initial behaviour in the pipe-rig system

In all six runs, the period from 0h (start of sampling) to 160h (first draining of system) was replicated. The total system output was collected, with sampling periods of 0h to 20h, 20h to 40h, 40h to 80h, 80h to 120h, and 120h to 160h. Oocyst counts from each of these samples were adjusted for recovery, and normalised per 20h residence time in the system. The logarithm of normalised count from each sampling period of the experimental replicates was then used to calculate mean (effectively geometric mean) and standard error values for each of the periods. These data have been presented in Figure 6, with the midpoint of each sampling period plotted against the mean oocyst count for the sample period, and error bars showing 2 standard errors about the mean.

For the purposes of comparison, Figure 6 also contains a theoretical plot of data expected should no surface interaction occur within the experimental pipe-rig system. The theoretical plot is to demonstrate the effect of oocyst sorption to surfaces within the system. It is calculated on the assumption that the system is operating as a chemostat, with initial inoculum, dilution rate and sampling periods as actually used. No testing was performed to ensure the theoretical model was appropriate for the experimental system. The following logarithmic decay equation was used to calculate oocysts remaining in the pipe-rig:

$$\text{Remaining oocysts} = \text{initial inoculum} * e^{(-\text{sampling time} / \text{residence time})}$$

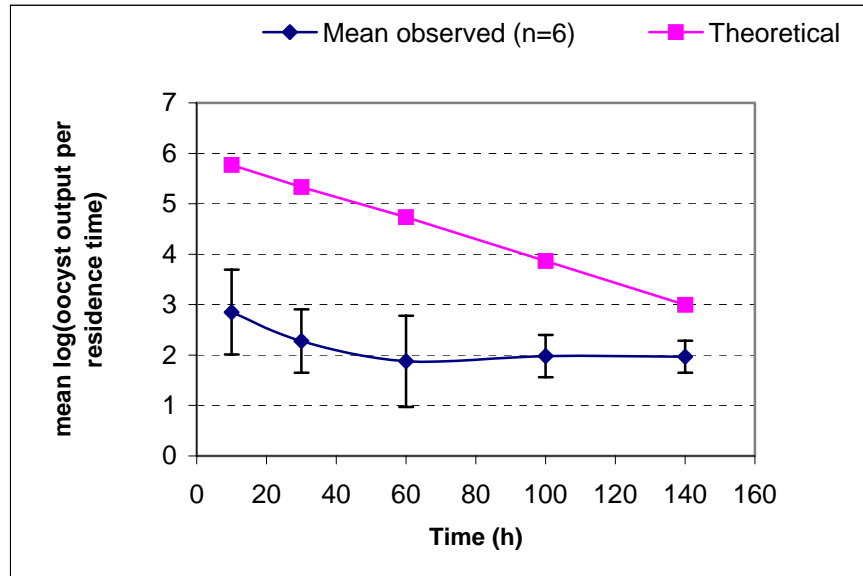


Figure 6: Oocyst output from system, 0h to 160h, compared to theoretical output with no surface interactions (Error bars = 2SE)

The initial inoculum value used was that observed from experimental data. Theoretical oocyst numbers output from the pipe-rig were calculated by subtracting expected number of oocysts in-system at the end of a time period from expected numbers at the beginning of a time period. This was then normalised to output per residence time. The results are shown below in Table 1, and are plotted in Figure 6.

Table 1: Expected oocyst output with no surface interaction

| Total time of collection | Theoretical oocyst output per 20h residence time |
|--------------------------|--|
| 0 to 20h | 580,000 |
| 20 to 40h | 210,000 |
| 40 to 80h | 54,000 |
| 80 to 120h | 7,300 |
| 120 to 160h | 990 |

The observed data shown in Figure 6 was significantly different from those expected from the theoretical plot where no surface interaction was assumed. This observation is consistent between the experimental replicates. The majority of oocysts inoculated into the system remained there, and were not washed out during this period of the experiment.

The observed oocyst output curve in Figure 6 demonstrates a two-stage process. The combined data points from 10 and 30h output differ significantly from the combined 60, 100 and 140h output (t-test, $p=0.04$). The curve is relatively steep from the beginning of output through to about the 60h mark, at which stage it becomes more flattened. This could be explained by initial washout of oocysts from the bulk water phase, followed by intermittent detachment over the remainder of the experiment.

System draining following initial conditions

Following 160 hours of sample output, the experimental system was emptied. This formed the “160h bulk” sample. This sample was processed in the same way as other water samples. The final part of the water drained from the system always contained a substantial quantity of resuspended material, which was not included in the 160h bulk sample to avoid subsequent concentration difficulties. This included what was subjectively estimated to be the final 500 to 1000mL of water in the system. The resuspended material appeared to be largely sloughed biofilm fragments, with approximate surface area of 4cm^2 or less. These fragments were thin and translucent, and were reddish-brown coloured, probably from incorporated corrosion products. Some samples were taken of this resuspended material, these formed “160h slurry” samples. A substantial amount of resuspended material could be expected to remain in the system following draining. Any process to recover it would have been destructive to biofilm remaining within the system. The recovered samples were concentrated using centrifugation, and then processed with IMS and examined by microscopy. Some fragments were mounted and fixed on microscope slides, stained with anti-*Cryptosporidium* IgG Cry104, and directly examined by microscopy.

Following the opening of the system, the internal surface of the pipe was sampled at Point A (Figure 1). This surface scraping was transferred to a 50mL centrifuge tube, and made up to a 10mL volume with sample buffer. This formed the “160h film” sample, and was processed with IMS and examined by microscopy.

The 160h bulk samples contained large numbers of inactivated oocysts ($>10^3$ oocyst/20L), usually about the same level as that observed in the combined sum of samples taken from 0h to 160h water samples (Appendix 1 and 3). Substantial variation was expected between the experimental iterations, due to the variable amount of resuspended material included from the final part of draining the system. A comparison of observed oocyst levels between the previous water samples and the 160h bulk sample is shown in Figure 7, as well as levels observed in later samples. Again, these figures have been corrected for recovery, and normalised to a count per volume of the experimental system. The draining process would remove water from any stagnant areas of the system, and could be expected to disturb loosely surface-attached material. This would explain the elevated oocyst levels observed in the bulk samples.

The biofilm and resuspended material samples also contained substantial levels of inactivated oocysts. These results are not directly comparable to those in the water phase. Observed data is tabulated below in Table 2. Results have been corrected for recovery, normalised for surface area or volume, and the mean of logarithmic values (geometric mean) used for subsequent calculations.

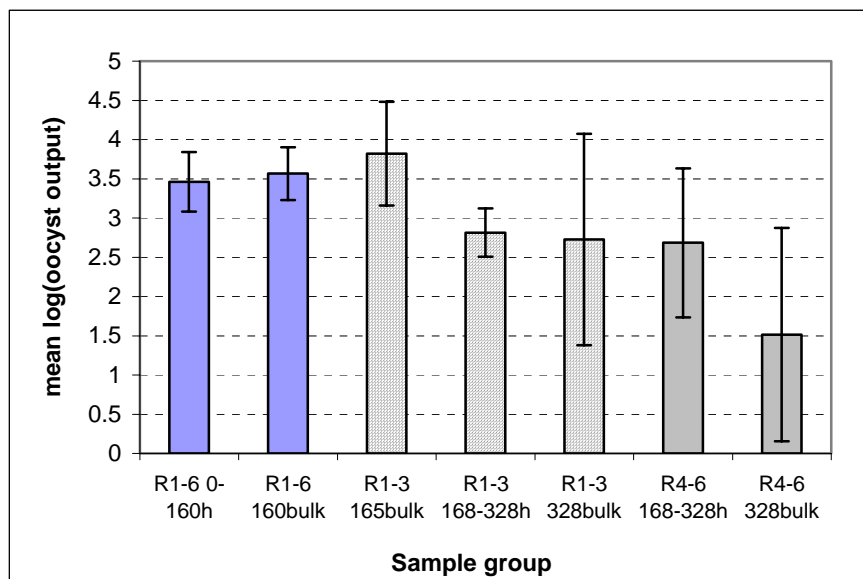


Figure 7: Total oocyst outputs from water samples (Error bars = 2SE)

Table 2: Oocyst numbers calculated from 160h bulk, biofilm and resuspended material

| Sample | Geomean oocysts per unit | Geomean oocysts in system |
|---------------------|--------------------------|---------------------------------|
| 160h “bulk” (n=6) | 3,680 per 20L | 3,680 per 20L |
| 160h “film” (n=6) | 1.17 per cm ² | 10,800 per 9,160cm ² |
| 160h “slurry” (n=3) | 7,680 per L | 7,680 per 1L |

The calculations of oocyst numbers assume that oocysts were homogenous in their distribution through the system, and that recovery efficiencies determined during analysis were accurate. This is examined further in the Discussion section.

Simulation of mains break and repair

This was performed in the second half of experimental runs 1, 2, and 3. Briefly, following the collection of the 160h “bulk” sample, the system was refilled with water containing 20mg/L chlorine. The system was left for 60 minutes, with water circulation provided in the last 5 minutes. The system was then drained and emptied as described above for post-initial conditions. Following determination of the chlorine concentration, the sample was dechlorinated with sodium thiosulfate. Samples were taken for the bulk water emptied from the system (“165h bulk”) and from surface scrapings at Point A (Figure 1; “165h film”). Concentrations observed are shown below in Table 3, and compared against other water output levels on Figure 7. The system was subsequently refilled with dechlorinated water, as described below.

The bulk water samples resulting from heavy chlorination showed marginally higher concentrations of oocysts than the previous, unchlorinated, bulk water samples. This relationship is statistically uncertain ($p=0.25$) as determined by the Wilcoxon signed-ranks test, due to the small number of observations for comparison ($n=3$). Despite the significant oocyst resuspension observed from this treatment, the majority of oocysts remained unaccounted for, presumably still attached within the experimental system.

The exact degree of reaction between the chlorinated water and the system biofilm was not determined, however the remaining chlorine in the bulk sample was on average 13.7 mg/L after 1h exposure, indicating substantial reaction. There was little shear stress during or subsequent to this reaction period, which would have reduced detachment phenomena.

Table 3: Oocyst numbers calculated from 165h bulk and biofilm

| Sample | Geomean oocysts per unit | Geomean oocysts in system |
|-------------------|--------------------------|---------------------------------|
| 165h "bulk" (n=3) | 6,620 per 20L | 6,620 per 20L |
| 165h "film" (n=3) | 1.30 per cm ² | 11,900 per 9,160cm ² |

At 168h, the experimental system was refilled with dechlorinated water, and water circulation and input were restarted. The total system output was collected, with sampling periods of 168h to 188h, 188h to 208h, 208h to 248h, 248h to 288h, and 288h to 328h. Oocyst counts from each of these samples were adjusted for recovery, and normalised per residence time in the system of 20h. The logarithm of normalised count from each sampling period of the experimental replicates were then used to calculate mean (effectively geometric mean) and standard error values for each of the periods. These data have been presented in Figure 8, with the midpoint of each sampling period plotted against, the mean logarithmic oocyst count, and error bars calculated for 2 standard errors.

Following the 288 to 328h sample, the system was emptied as described below in "End-point sampling for both simulations".

Simulation of upstream reservoir chlorination

This was performed in experimental runs 4, 5, and 6. At 168h, the experimental system was refilled with water containing 2mg/L of chlorine. The total system output was collected, with sampling periods of 168h to 188h, 188h to 208h, 208h to 248h, 248h to 288h, and 288h to 328h. Oocyst counts from each of these samples were adjusted for recovery, and normalised per residence time in the system of 20h. The logarithm of normalised count from each sampling period of the experimental replicates were then used to calculate mean (effectively geometric mean) and standard error values for each of the periods. These data have been presented in Figure 8, with the time value calculated from the midpoint

of each sampling period, the mean logarithmic oocyst count plotted, and error bars calculated using standard error.

In Figure 8, the dechlorinated and 2mg/L chlorinated input conditions (from the mains break – repair and upstream reservoir chlorination simulations, respectively) can be seen to be similar with respect to numbers of oocysts output from the system. When the mean of log-transformed values for each treatment is compared using the Wilcoxon signed-ranks test, neither mean is statistically larger than the other ($p > 0.2$ in both cases).

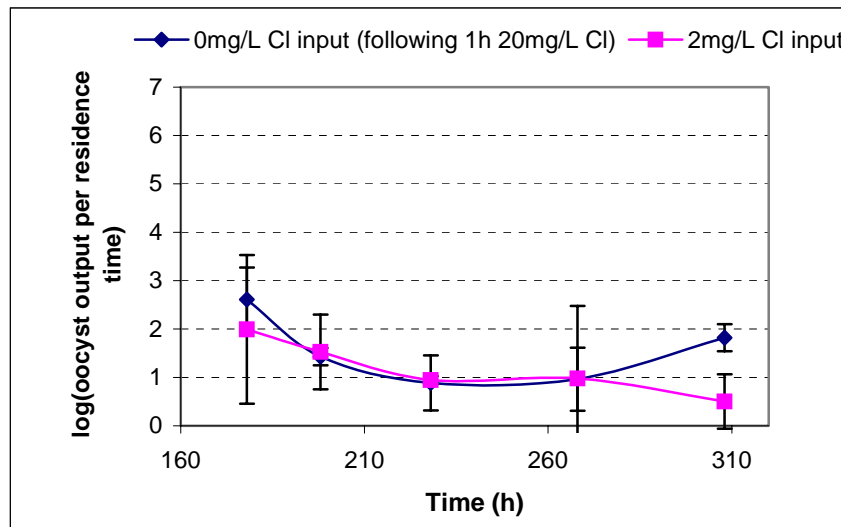


Figure 8: Oocyst output from system, 168h to 328h (Error bars = 2SE)

There is no particular pattern of oocyst output over time observed in Figure 8, and the amount of variation within time periods is large. All oocysts released during this period of the experimental runs had been surface associated, remaining after the earlier draining of the system. Rate and timing of oocyst detachment, and subsequent release from the system under the tested experimental conditions, may be essentially random. Total oocyst numbers released over this period are compared with other output periods on Figure 8.

The runs with 2mg/L chlorine input produced output samples averaging 0.05mg/L chlorine (standard error 0.008). It is apparent that this level of chlorine was consumed relatively rapidly within the system, and in doing so did not substantially affect oocyst detachment rates.

Following the 288 to 328h sample, the system was emptied as described below in “End-point sampling for both simulations”.

End-point sampling for both simulations

Following the second 160 hour period of sample output, the experimental system was emptied. This formed the “328h bulk” sample. This sample was processed in the same way as other water samples. Resuspended material was treated as described above in “System draining following initial

conditions”, when sampled this material formed “328h slurry” samples. Surface scrapings were taken at Point A (Figure 1), this formed a “328h film” sample. Additionally, a second sample was taken at Point B (Figure 1), to provide a basis of comparison of variation within the system. This was referred to as “328h film – 2nd sample”.

The 328h bulk samples frequently contained large numbers of oocysts (inactivated), although a very large amount of variation between the experimental runs was observed. This can be seen in the comparison in Figure 7 against the variation observed in other sample groups from the system. As with the other bulk samples, variation was expected between the experimental iterations, due to the variable amount of resuspended material included from the final part of draining the system.

The biofilm and resuspended material samples also contained oocysts (inactivated). These results are not directly comparable to those in the water phase. Observed data is tabulated below in Table 4. Results have been corrected for recovery, normalised for surface area or volume, and the logarithmic value used for subsequent calculations.

Table 4: Oocyst numbers calculated from 328h bulk, biofilm and resuspended material

| Sample | Geomean oocysts per unit | Geomean oocysts in system |
|---|---------------------------|--------------------------------|
| <i>From Runs 1-3 (unchlorinated)</i> | | |
| 328h “bulk” (n=3) | 533 per 20L | 533 per 20L |
| 328h “film” (n=3) | 0.908 per cm ² | 8,320 per 9,160cm ² |
| 328h “slurry” (n=2) | 862 per L | 862 per 1L |
| <i>From Runs 4-6 (2mg/L chlorine input)</i> | | |
| 328h “bulk” (n=3) | 32.7 per 20L | 32.7 per 20L |
| 328h “film” (n=3) | 0.425 per cm ² | 3,890 per 9,160cm ² |
| 328h “slurry” (n=3) | 7,530 per L | 7,530 per 1L |

These results assume that oocysts are homogenous in their distribution through the system, and that recovery efficiencies determined during analysis were accurate. This is examined further in the Discussion section.

An indication of the variation of attachment to surfaces within the system was calculated by comparison of the “biofilm” and the “biofilm – 2nd sample” oocyst counts (sampled at points A and B,

respectively; Figure 1) of the same runs, tabulated in Appendix 1. The values from point A were used in other analyses, this was the point sampled at other time periods as well. Flow prior to this point was along a straight section of pipe. Point B was only sampled at 328h, the data generated was not used in other comparisons. Flow before point B was around the bend section of the pipe loop.

The matched pairs of log-transformed data were examined using the Wilcoxon signed-ranks test (Helsel and Hirsch, 1992), weakly showing the matched oocyst counts at point B were larger than at point A at the same time of sampling ($p=0.062$), this comparison limited by the small sample number.

Estimated total outputs for the tested systems are presented in Appendix 3.

3.3 Supplementary data

The data from the supplementary testing (temperature, pH, HPCs, chlorine) have been summarised from Appendix 2 and presented graphically below in Figures 9 to 13. Observed data for these parameters was similar between experimental replicates, some variation was observed as shown by the error bars (2 standard errors) in these figures.

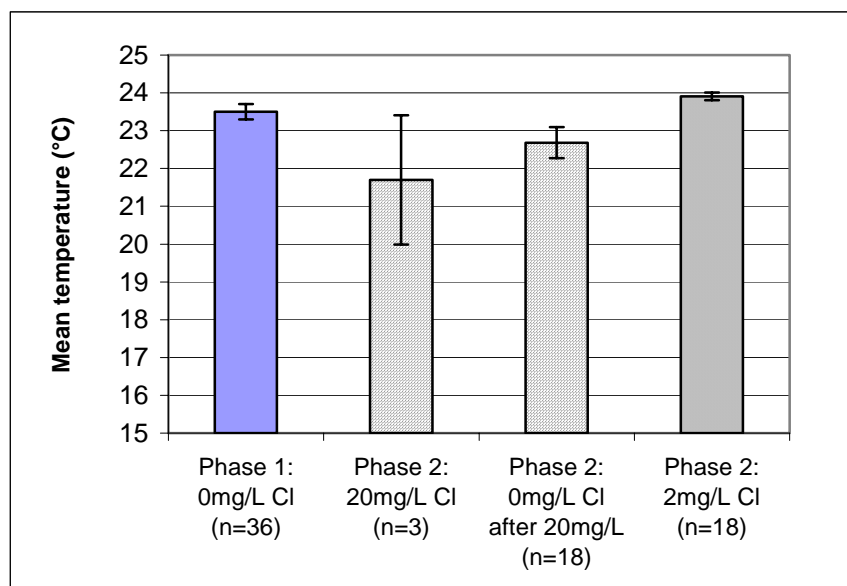


Figure 9: Temperature Variation (Error bars = 2SE)

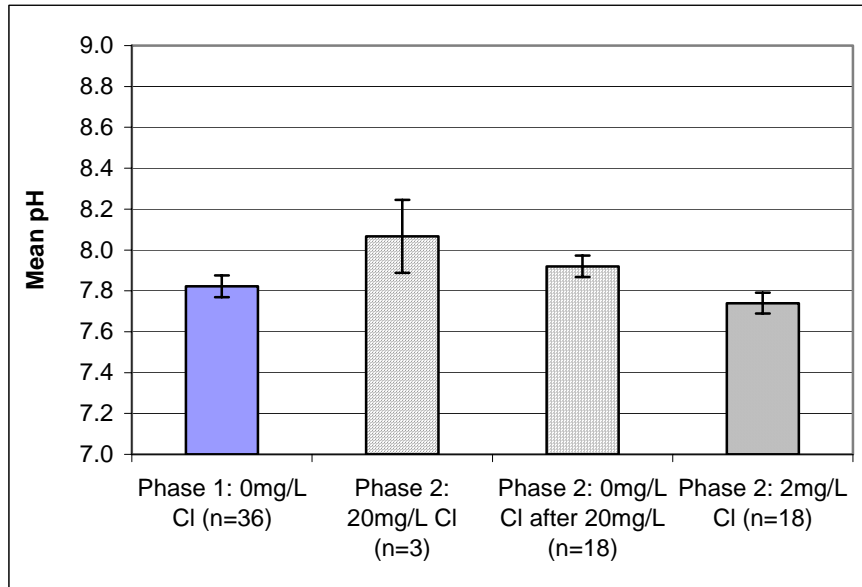


Figure 10: pH Variation (Error bars = 2SE)

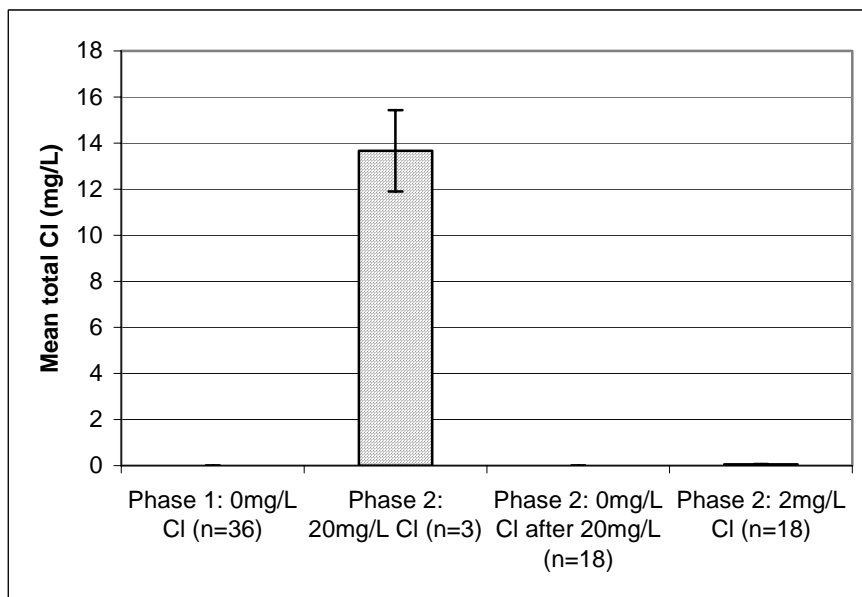


Figure 11: Chlorine Variation (Error bars = 2SE)

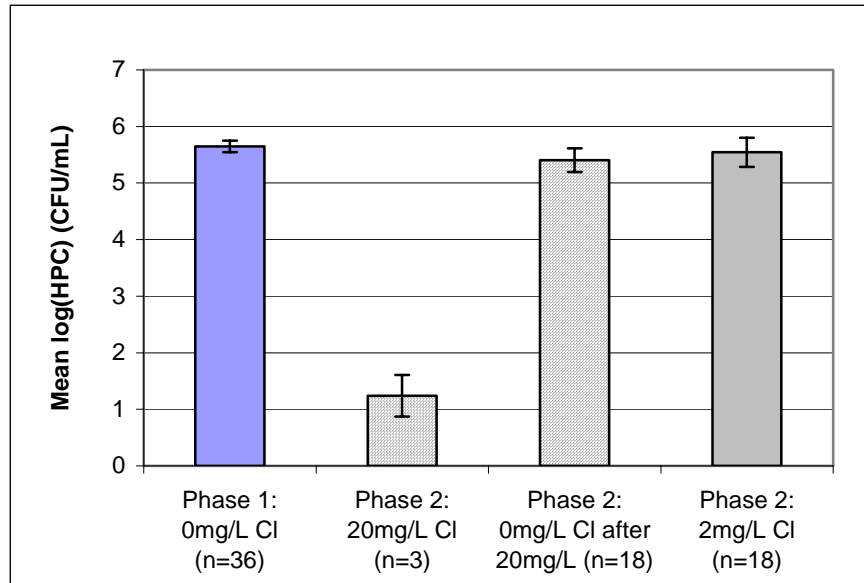


Figure 12: HPC Variation in water (Error bars = 2SE)

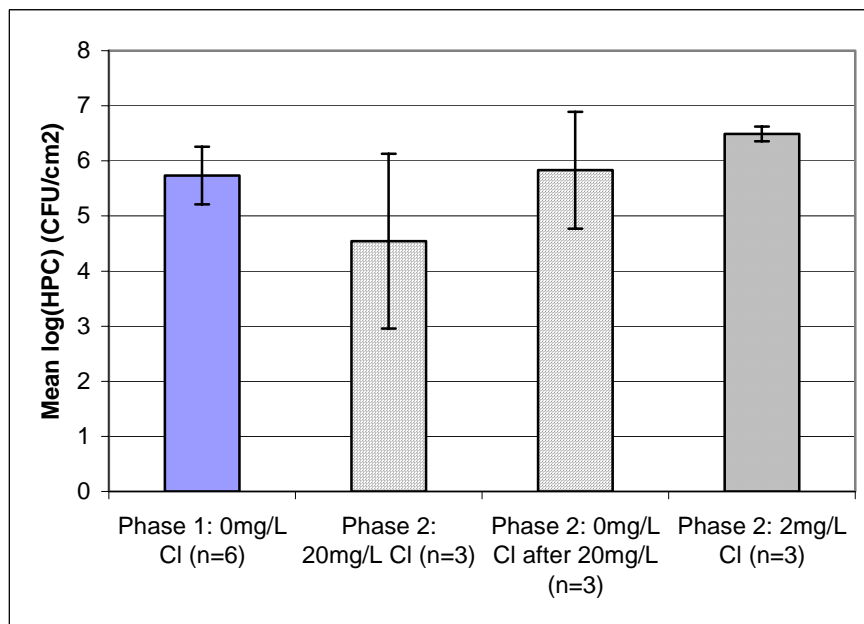


Figure 13: HPC Variation in biofilm (Error bars = 2SE)

Of the water samples taken in the second phase of the experimental runs, heterotrophic plate counts in the systems with an input of 2mg/L of chlorine were similar to systems with no chlorine input (Figure 12). Furthermore, heterotrophic plate count concentrations in the water phase appeared to be relatively constant in all systems with 0 to 2mg/L chlorine input (Figures 12), which indicated a similar level of bacterial activity between experimental runs.

The average total chlorine level measured in these output samples was 0.05mg/L. The HPC levels in the chlorinated systems' biofilm samples appeared to be slightly higher (geometric mean increased by 0.7 log), although this is within the bounds of experimental error.

Heavy (20mg/L) chlorination for 1h resulted in a decline in the geometric mean of HPCs of approximately 4 logs in the water phase (Figure 12), geometric mean of HPCs in biofilm samples declined by 1 to 1.5 logs from this exposure (Figure 13). However, the biofilm samples showed extensive variation between samples (Figure 13). Samples taken from the system following heavy chlorination showed substantial chlorine demand, with an average of 13.7mg/L chlorine residual observed (Figure 11).

The chlorinated runs were performed at slightly higher temperatures than the non-chlorinated system (23.9°C compared to 22.7°C, 2 standard errors of 0.10°C and 0.42°C, respectively; Figure 9). The pH levels of the chlorinated system were on average marginally lower than the non-chlorinated system (7.7 compared to 7.9, 2 standard errors of 0.06 in both cases; Figure 10). This was attributed to variation in the source tap water over the period of experimentation; it was thought this variation would not greatly affect results.

4. Discussion

4.1 Surface attachment of *Cryptosporidium* oocysts

The majority of *Cryptosporidium* oocysts introduced into the pipe-rig system were not subsequently recovered in water displaced from the pipe-rig. See Appendix 3 for estimates of total outputs and retained oocysts.

It is likely that the oocysts not accounted for in the pipe-rig water samples were attached to surfaces within the system. Distribution on different surfaces within the system would appear to be variable, although only a limited number of areas of the system were examined due to time and resource constraints. The hydrodynamics of the experimental system were not examined; it is possible that non-sampled areas of reduced flow would exist in some parts of the system. Such areas could have accumulated a large proportion of the inoculated oocysts.

The recovery of oocysts in water samples was fairly constant, with mean recovery of 41% (standard deviation 11%, n=6) applied to all samples. As recovery from surface scrapings and resuspended material was quite variable (mean recovery 54%, standard deviation 22%, n=20), a positive control sample was performed with each of these samples, and the recovery efficiency calculated for each sample. Although recovery from these control samples appeared to be quite efficient, this was using oocysts seeded shortly before the IMS separation process; while those within the sample matrix are likely to be more difficult to recover.

Some oocyst losses from disintegration within the system could also be expected. Although regarded as environmentally robust, oocyst losses over time in this fashion may have accounted for a large proportion of the inoculum. As the system was initially dechlorinated and microbiologically active, biological effects such as bacterial secretion of exoenzymes within the biofilm may have substantially contributed to oocyst disintegration. Grazing from free-living protozoa may also have resulted in oocyst losses, these organisms would not have been detected in the assays used. Further experimentation under more controlled conditions, such as in an annular reactor, would be necessary to determine the level of biological effects.

Recovery data generated from seeding pipe-rig water can be regarded as accurate, lending confidence to the oocyst levels observed in the water samples. The detection method used is routinely applied to water samples, and can expect recoveries as observed from turbid water samples. Processing losses can be attributed primarily to incomplete elution and collection after the membrane concentration step, and from the process of breaking up clumped samples by syringing through mesh. The seeding and recovery process incorporated all the steps where losses would have occurred to an actual sample. Large unobserved losses in detection can be regarded as unlikely, with generally repeatable oocyst numbers observed in water samples from the different experimental runs, at least

before the proposed intermittent surface detachment becomes the major factor determining suspended oocyst numbers. Variation was observed between the runs, but not at levels suggesting large variation in recovery efficiency.

4.2 Subsequent detachment of *Cryptosporidium* oocysts

Cryptosporidium oocyst results from pipe-rig water samples did not provide information on numbers of surface-attached oocysts. Some correlation could have been expected if there was a constant rate of oocyst detachment, or if a dynamic equilibrium existed between attached and non-attached oocysts.

Contaminated pipe surfaces were shown to be capable of intermittent release of oocysts over an extended period of time. This was shown with the numbers of oocysts observed in water samples taken from refilled pipes, and the variability of oocyst numbers over time in these samples. There were no changes in experimental conditions over these periods of intermittent release. This resuspension was particularly evident in pipe-rig discharge water samples after two residence periods in the pipe loop (40h), following substantial washout of the initial inoculum (as shown in Figure 6).

The level of oocysts detected in “bulk” samples, when the pipe system was drained and examined, was frequently and substantially greater than the previous pipe-rig water sample. This may be explained by greater shear stresses generated in collection of the bulk sample, resulting in increased resuspension of oocysts. However, an increase in recovered numbers was not always observed. Although the samples were collected in the same way, the amount of resuspension may be quite variable between experiments.

A quantity of resuspended biofilm was observed when emptying the pipe-rig system. Following direct microscopic examination, this biofilm showed some attached oocysts. Quantitative examination using IMS showed substantial quantities of oocysts present in this resuspended material. Recovery of oocysts seeded to this material immediately prior to IMS was variable, but generally good. However, the recovery of oocysts embedded in an EPS matrix or attached to particulates is likely to be lower than reported; with substantial numbers of oocysts remaining embedded or attached, and therefore undetected. An improved oocyst extraction method for these samples would be desirable.

It is likely that a substantial amount of resuspended biofilm was left within the system during sample collection and opening of the pipe. This could include a large proportion of oocysts not recovered from other samples.

4.3 Experimental system

There is no possible “typical” pipe system, which can be simulated, due to the great range of conditions present in a complete distribution system. The pipe-rig test system used can be regarded as having representative surface conditions of an in-service pipe, being primarily composed of exhumed pipe. The low shear stresses and disinfectant residual used, while uncommon, could be found in the end-reaches or a “dead-end” of a distribution system.

The method of oocyst dosing into the system was the introduction of a single large dose, followed by gradual dilution in low-flow and low-shear conditions. This would simulate the mode of introduction following an isolated contamination event within the distribution system. A scenario of a pulse of oocysts passing through a treatment plant in a breakthrough event would not be entirely dissimilar to this, although would involve different environmental conditions to those used here, and the oocyst surface characteristics would probably be modified by water treatment processes. A more gradual and extended release of oocysts from a treatment plant would be less similar to the conditions used in this study.

The experimental conditions appeared to be similar between the replicated experimental runs. This was the case with temperature, pH, chlorine concentration and heterotrophic plate counts. Oocyst levels, although generally similar between replicated runs, showed more variation.

A theoretical model of the experimental system as a chemostat with no surface interaction showed substantially different oocyst outputs to those observed experimentally, as shown in Figure 6. The difference between theoretical and observed behaviour would be explained by the majority of oocysts initially attaching to surfaces, with subsequent intermittent detachment. This may explain the 2-stage process observed in Figure 6, with an initial steep decline resulting from oocyst wash-out from the system, followed by a relatively flat curve due to intermittent release. Decay and disintegration of *Cryptosporidium* has not been incorporated into the theoretical plot, this would make the decrease of oocysts in the output occur at a steeper gradient. Additionally, the reactor flow conditions have not been validated to ideal chemostat performance, deviation from which would provide another source of error.

4.4 Effects of chlorination

Two chlorination regimes were performed during the experiments. The first attempted to simulate disinfectant conditions following the repair of a mains break, which could result in an input of pathogens to a distribution system. This included a 1h period where 20mg/L of chlorine was held within the pipe under no-flow conditions, followed by removal of disinfectant, and renewal of normal supply at 0mg/L. The second regime was an attempt to simulate conditions following the chlorination of an upstream reservoir, which may be performed following evidence of contamination of that reservoir. This involved raising the input chlorine concentration to 2mg/L. Both of these regimes followed initial conditions of one week running at 0mg/L chlorine, oocyst inoculation, and then another week at 0mg/L.

High dose chlorination (20mg/L) for 1h resulted in substantial release of oocysts from a contaminated pipe surface, although the majority of oocysts remained attached to the surface. A substantial chlorine demand was demonstrated within the system during the chlorination process.

Continuous dose chlorination at 2mg/L in this system resulted in almost complete reduction of chlorine in the water phase. Small numbers of oocysts were resuspended in the water phase while this level of

chlorine was applied, although these numbers were not significantly different to the unchlorinated system. This level of chlorination did not result in large-scale resuspension of oocysts from the pipe biofilm, which may have resulted from biofilm modification caused by reducing chlorine.

The chlorine exposure conditions in this experiment (20mg/L for 1h, and 2mg/L for up to 40h) should not have affected detection of oocysts. Previous internal method development work (unpublished) has shown that exposure of greater than 20mg/L for 20h was required prior to loss of detection. This work also found the exposure level used in cleaning of the pipes elbow and end caps (50mg/L for 24h) was adequate to prevent detection of oocysts and therefore avoid carryover between experiments.

4.5 Application to distribution systems

Outcomes from this study have shown that if the surface attachment and subsequent intermittent detachment of oocysts were a major factor in actual field conditions, variability between samples over time would be high and would provide little benefit in terms of risk assessment. This indicates that routine sampling of distribution systems for the presence of *Cryptosporidium* is of little value and supports a risk-based approach to managing drinking water quality, as promulgated by the Framework for Management of Drinking Water Quality (NHMRC, 2004) and the WHO Guidelines for Drinking Water Quality (WHO, 2004). Under the risk-based approach, focus should be placed on preventing oocysts from entering distribution systems by optimising treatment processes and implementing effective catchment management. Nevertheless, under incident conditions where oocysts may enter a distribution system, samples taken over a range of time or space would be of greater value than samples taken at specific time periods, given the likely variability between samples. Such composite samples could include continuous sampling and concentration devices such as cartridges or filter columns, or samples concentrated by treatment processes such as filter backwashes, and combined samples from multiple points of a distribution system. Areas of a system containing “dirty” water from resuspension of sediments or biofilms would also be of particular interest, examination would require sound methods able to examine turbid waters, surface scrapings and particulates. These could include, amongst others, “dead-end” sections of pipes and reservoir sediments. It should be noted that resuspended oocysts are likely to be older and potentially less viable than oocysts directly transported in the bulk phase, which may affect interpretation of results.

The value of other ad-hoc biofilm and sediment samples taken from a distribution system is uncertain. The current difficulties in oocyst recovery from these matrices, and the observed variability of oocyst distribution due to flow dynamics within the pipe would make such observations of limited value.

The interaction of shear stress and oocyst detachment and resuspension remains incompletely understood. In application to a distribution system compromised by *Cryptosporidium*, processes such as flushing may physically remove oocysts from a system, but may also result in subsequent increased oocyst numbers in the bulk phase from biofilm and sediment disturbance. To allow for this, flushing should continue until disturbed particles are removed from the pipes, and flushing programs

designed so that sediments and detached biofilms are removed with the flushed water rather than redistributed to other sections of the distribution system.

Due to safety considerations, this study used inactivated oocysts. It is unclear, therefore, as to how long biofilm associated oocysts remain viable, and once non-viable how long they can be detected. Attachment would reduce exposure to disinfectants, but over the long term would result in lengthy contact times. Improved understanding of these issues would assist in risk management.

Chlorine introduced at an input level of 2mg/L was predominantly consumed within the system. This process did not result in an increased rate of oocyst detachment. Should this apply to distribution system conditions, then disinfection alone should not result in oocyst resuspension.

Exposure to 20mg/L chlorine as a pipe cleaning process can be expected to remove substantial numbers of oocysts attached to a pipe wall. Shear effects during a cleaning process should also contribute to detachment, although no data is currently available to quantify this.

Given the apparent difficulties in generating meaningful data to assess *Cryptosporidium* through the system, a premium should be placed on the processes of catchment protection, treatment optimisation, and programs to ensure the integrity and maintenance of the distribution system as proposed by risk-based approaches to managing drinking water quality (NHMRC, 2004; WHO, 2004).

4.6 Directions of further study

Desirable technical advances would include improved isolation of *Cryptosporidium* from surface materials such as biofilm or sediments. The method used in this study demonstrated good recoveries, but may have been affected by the particulate load of the samples and by the presence of iron corrosion products. Additionally, as recoveries were calculated using oocysts inoculated prior to processing, actual recoveries of oocysts embedded within biofilm matrices or attached to particulates may have been lower. Further improvements and combined application of advanced separation methods appears to offer the best chance of adequate recoveries. This may include combining methods such as flow cytometry and immunomagnetic separation, and use of acid or enzyme digestion processes to remove metal oxides and polysaccharides from samples.

The separation of oocysts from environmental matrices remains as the largest obstacle to accurate detection and enumeration. Samples were processed with extensive vortex mixing, which may have been inadequate to separate oocysts embedded in large amounts of biofilm material. Homogenisation of oocyst-seeded suspended biofilm samples was attempted, but resulted in very poor recoveries (data not shown). This method was not employed in processing of experimental samples.

There needs to be better understanding of the effects of shear stresses on oocyst attachment and detachment, and of the effects on oocyst viability and detection resulting from attachment. These areas were not examined in this study, due to the perceived unsuitability of this particular experimental system for these other investigations. An investigation of shear stress would be best performed using a system in which shear can be more precisely controlled, such as an annular reactor. This sort of

system would allow repeated examination of surface samples using removable slides, and provided that biological and surface characteristics could be mimicked would also be suitable for viability and detection studies. It may be necessary to use a combination of experimental systems to adequately describe these processes.

5. Summary and Conclusions

The majority of inactivated *Cryptosporidium* oocysts introduced into the experimental pipe-rig system were not subsequently recovered in pipe-rig effluent samples. These oocysts were most likely heterogeneously attached to particles and surfaces through the system. Some disintegration of oocysts within the system was also possible. Attached oocysts were difficult to recover from surface materials.

Results from water samples did not provide information on numbers of surface-attached oocysts. Contaminated surfaces in the pipe-rig were shown to be capable of intermittent release of oocysts over an extended period of time. Resuspended biofilm was observed when emptying the pipe-rig system; this material was shown to contain substantial numbers of (inactivated) oocysts.

High dose chlorination (20mg/L) for 1h resulted in substantial release of oocysts from a contaminated pipe surface, although the majority of oocysts apparently remained attached to the surface. No significant increase in oocyst resuspension was observed following continuous input of 2mg/L chlorine, compared to 0mg/L chlorine. A large chlorine demand was observed in all dosing studies.

The results from the study support a risk-based approach to the management of drinking water quality, with focus on effective source water catchment management and optimised treatment to prevent *Cryptosporidium* oocysts from entering drinking water supplies. Under incident conditions, where oocysts may contaminate a drinking water supply, should surface attachment and subsequent intermittent detachment be a major factor in actual field conditions, variability between samples over time would be high. In this case, composite samples would be of more value than grab samples. Areas of a system containing “dirty” water from resuspension of sediments or biofilms would also be of interest. The value of other ad-hoc biofilm and sediment samples taken from a distribution system is uncertain, due to expected heterogenous distribution and limitations of detection methods.

Prediction of behaviour in the distribution system requires greater understanding of the effects of shear stresses on oocyst attachment and detachment, and of the effects on oocyst viability and detection resulting from attachment. Such understanding requires a more substantial project than this one, using more closely controlled experimental conditions and ready access to surfaces.

6. Recommendations

The following recommendations are suggested by the project outcomes.

- It is recommended that routine sampling of distribution systems not be undertaken. This is based on the observed large variation in oocyst concentration between grab water samples from intermittent oocyst detachment. Rather a risk-based approach to managing drinking water quality should be adopted with focus on effective catchment management and optimisation of treatment processes to prevent oocysts from entering the distribution system.
- Under incident conditions, where distribution system contamination may occur, sampling should either be as continuous sampling or composite sampling. This is based on the observed large variation in oocyst concentration between grab water samples from intermittent detachment.
- It is recommended that ad-hoc monitoring of biofilms and sediment not be undertaken due to the inherent heterogeneity of surface incorporation in drinking water pipe biofilms. If necessary, monitoring of biofilms and sediment should be undertaken in a structured fashion taking into account system heterogeneity.
- It is recommended that further research be undertaken to determine the fate of oocysts in distribution systems with specific reference to oocyst viability and persistence. This will better address risk management approaches to water quality management.

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Appendix 1

Transformed oocyst data

All results expressed to 3 significant figures.

Transformed results, water samples

| Sample period | | Run 1 | Run 2 | Run 3 | Run 4 | Run 5 | Run 6 |
|---------------|----------|-------|-------|-------|-------|-------|-------|
| 0-20h | Count/RT | 380 | 12.1 | 7750 | 3300 | 3490 | 317 |
| | Log(#) | 2.58 | 1.08 | 3.89 | 3.52 | 3.54 | 2.50 |
| 20-40h | Count/RT | 67.8 | 12.1 | 249 | 1760 | 172 | 765 |
| | Log(#) | 1.83 | 1.08 | 2.40 | 3.24 | 2.24 | 2.88 |
| 40-80h | Count/RT | 626 | 8.47 | 183 | 460 | 333 | 1.21 |
| | Log(#) | 2.80 | 0.928 | 2.26 | 2.66 | 2.52 | 0.08 |
| 80-120h | Count/RT | 249 | 50.8 | 63.0 | 213 | 14.5 | 299 |
| | Log(#) | 2.40 | 1.71 | 1.80 | 2.33 | 1.16 | 2.48 |
| 120-160h | Count/RT | 167 | 149 | 20.6 | 44.8 | 165 | 166 |
| | Log(#) | 2.22 | 2.17 | 1.31 | 1.65 | 2.22 | 2.22 |
| 168-188h | Count/RT | 596 | 293 | 390 | 4.84 | 2190 | 89.6 |
| | Log(#) | 2.77 | 2.47 | 2.59 | 0.685 | 3.34 | 1.95 |
| 188-208h | Count/RT | 48.4 | 7.26 | 55.7 | 33.9 | 7.26 | 157 |
| | Log(#) | 1.69 | 0.861 | 1.75 | 1.53 | 0.861 | 2.20 |
| 208-248h | Count/RT | 31.5 | 2.42 | 6.05 | 7.26 | 9.69 | 9.69 |
| | Log(#) | 1.50 | 0.384 | 0.782 | 0.861 | 0.986 | 0.986 |
| 248-288h | Count/RT | 4.84 | 12.1 | 13.3 | 1.21 | 2.42 | 292 |
| | Log(#) | 0.685 | 1.08 | 1.12 | 0.083 | 0.384 | 2.47 |
| 288-328h | Count/RT | 264 | 18.2 | 60.5 | 1.21 | 2.42 | 10.9 |
| | Log(#) | 2.42 | 1.26 | 1.78 | 0.083 | 0.384 | 1.04 |

“Count/RT” is the oocyst count for the sample, modified for recovery (41%), and expressed per residence time (20L).

“Log(#)” is the logarithm of the count/residence time.

Transformed results, bulk water samples

| Sample | | Run 1 | Run 2 | Run 3 | Run 4 | Run 5 | Run 6 |
|-----------|-----------|-------|-------|-------|-------|-------|-------|
| 160h bulk | Count/20L | 17900 | 3810 | 1020 | 2200 | 3460 | 4720 |
| | Log(#) | 4.25 | 3.58 | 3.01 | 3.34 | 3.54 | 3.67 |
| 165h bulk | Count/20L | 30300 | 3270 | 2930 | N/A | N/A | N/A |
| | Log(#) | 4.48 | 3.51 | 3.50 | | | |
| 328h bulk | Count/20L | 2060 | 24.2 | 3030 | 2.42 | 26.6 | 540 |
| | Log(#) | 3.31 | 1.38 | 3.48 | 0.384 | 1.43 | 2.73 |

“Count/20L” is the oocyst count for the 20L sample, modified for recovery (41%).

“Log(#)” is the logarithm of the count /20L.

Transformed oocyst data, biofilm samples

| Sample | | Run 1 | Run 2 | Run 3 | Run 4 | Run 5 | Run 6 |
|-------------------------------------|-----------------------|-------|-------|-------|-------|-------|-------|
| 160h film | Recovery | 0.83 | 0.08 | 0.41 | 0.53 | 0.58 | 0.32 |
| | Count/cm ² | 19.1 | 1.05 | 0.391 | 0.303 | 1.11 | 0.993 |
| | Log(#)/all | 5.24 | 3.98 | 3.55 | 3.44 | 4.01 | 3.96 |
| 165h film | Recovery | 0.77 | 0.69 | 0.64 | N/A | N/A | N/A |
| | Count/cm ² | 9.34 | 0.232 | 1.00 | | | |
| | Log(#)/all | 4.93 | 3.33 | 3.96 | | | |
| 328h film | Recovery | 0.67 | 0.14 | 0.57 | 0.39 | 0.62 | 0.66 |
| | Count/cm ² | 0.477 | 1.40 | 1.12 | 0.411 | 0.256 | 0.729 |
| | Log(#)/all | 3.64 | 4.11 | 4.01 | 3.58 | 3.37 | 3.82 |
| 328h film 2 nd Sample | Recovery | 0.22 | NP | 0.47 | 0.65 | 0.89 | 0.63 |
| | Count/cm ² | 1.43 | | 0.684 | 0.736 | 4.33 | 3.56 |
| | Log(#)/all | 4.12 | | 3.80 | 3.83 | 4.60 | 4.51 |

"Recovery" was calculated from a parallel spiked sample (1.00=100%).

"Count/cm²" is the IFA oocyst count modified by the recovery figure, and expressed per cm² of sample examined.

"Log(#)/all" is the logarithm of the oocyst count extrapolated to the whole system (log(9160 x count/cm²)).

N/A: Not applicable; NP: Not performed

Transformed oocyst data, resuspended biofilm samples

| Sample | | Run 1 | Run 2 | Run 3 | Run 4 | Run 5 | Run 6 |
|-------------|----------|-------|-------|-------|-------|-------|-------|
| 160h slurry | Recovery | NP | NP | NP | 0.46 | 0.95 | 0.89 |
| | Count/L | | | | 20800 | 1680 | 13000 |
| | Log(#)/L | | | | 4.32 | 3.22 | 4.11 |
| 328h slurry | Recovery | NP | 0.48 | 0.13 | 0.32 | 0.79 | 0.88 |
| | Count/L | | 166 | 4470 | 6650 | 6040 | 10600 |
| | Log(#)/L | | 2.22 | 3.65 | 3.82 | 3.78 | 4.03 |

“Recovery” was calculated from a parallel spiked sample (1.00=100%).

“Count/L” is the IFA oocyst count modified by the recovery figure, and expressed per L of sample examined.

“Log(#)/L” is the logarithm of the oocyst count per L.

NP: Not performed

Appendix 2

Supplementary data

Temperature (°C)

| Sample | Run 1 | Run 2 | Run 3 | Run 4 | Run 5 | Run 6 |
|-----------|-------|-------|-------|-------|-------|-------|
| 0-20h | 23.0 | 22.8 | 22.5 | 23.8 | 24.6 | 24.0 |
| 20-40h | 24.0 | 23.2 | 23.0 | 23.5 | 24.3 | 23.8 |
| 40-80h | 23.7 | 23.0 | 23.0 | 24.0 | 24.0 | 24.2 |
| 80-120h | 22.9 | 22.7 | 22.8 | 24.0 | 23.7 | 24.0 |
| 120-160h | 22.4 | 22.9 | 23.5 | 23.9 | 24.2 | 24.0 |
| 160h bulk | 22.4 | 23.2 | 23.0 | 23.9 | 24.3 | 23.9 |
| 165h bulk | 20.7 | 21.0 | 23.4 | N/A | N/A | N/A |
| 168-188h | 21.7 | 22.8 | 23.6 | 23.8 | 23.8 | 23.9 |
| 188-208h | 21.0 | 23.1 | 23.4 | 23.8 | 23.8 | 24.4 |
| 208-248h | 21.9 | 23.2 | 23.8 | 23.5 | 24.0 | 24.1 |
| 248-288h | 21.4 | 22.8 | 23.2 | 23.8 | 23.8 | 23.5 |
| 288-328h | 21.7 | 22.6 | 23.3 | 23.9 | 24.1 | 24.0 |
| 328h bulk | 21.8 | 23.2 | 23.8 | 23.9 | 24.1 | 24.1 |

N/A: Not applicable

pH

| Sample | Run 1 | Run 2 | Run 3 | Run 4 | Run 5 | Run 6 |
|-----------|-------|-------|-------|-------|-------|-------|
| 0-20h | 8.03 | 7.85 | 8.02 | 7.83 | 7.63 | 7.72 |
| 20-40h | 8.14 | 7.74 | 7.93 | 7.72 | 7.59 | 7.77 |
| 40-80h | 8.01 | 7.99 | 7.95 | 7.85 | 7.64 | 7.72 |
| 80-120h | 8.01 | 8.00 | 7.85 | 7.75 | 7.61 | 7.68 |
| 120-160h | 8.04 | 7.83 | 7.93 | 7.69 | 7.63 | 7.70 |
| 160h bulk | 8.10 | 7.79 | 7.88 | 7.70 | 7.54 | 7.72 |
| 165h bulk | 8.18 | 8.13 | 7.89 | N/A | N/A | N/A |
| 168-188h | 8.09 | 7.75 | 7.89 | 7.75 | 7.59 | 7.71 |
| 188-208h | 8.01 | 7.92 | 7.98 | 7.96 | 7.61 | 7.68 |
| 208-248h | 8.00 | 7.88 | 7.95 | 7.70 | 7.62 | 7.77 |
| 248-288h | 8.04 | 7.83 | 7.77 | 7.87 | 7.67 | 7.76 |
| 288-328h | 8.05 | 7.84 | 7.79 | 7.86 | 7.67 | 7.78 |
| 328h bulk | 8.09 | 7.83 | 7.85 | 7.73 | 7.65 | 7.94 |

N/A: Not applicable

Total Chlorine (mg/L)

| Sample | Run 1 | Run 2 | Run 3 | Run 4 | Run 5 | Run 6 |
|-----------|-------|-------|-------|-------|-------|-------|
| 0-20h | 0 | 0 | 0 | 0 | 0 | 0 |
| 20-40h | 0 | 0 | 0 | 0 | 0 | 0 |
| 40-80h | 0 | 0 | 0 | 0 | 0 | 0 |
| 80-120h | 0 | 0 | 0 | 0 | 0 | 0 |
| 120-160h | 0 | 0 | 0 | 0 | 0 | 0 |
| 160h bulk | 0 | 0 | 0 | 0 | 0 | 0 |
| 165h bulk | 15 | 12 | 14 | N/A | N/A | N/A |
| 168-188h | 0 | 0 | 0 | 0.08 | 0.06 | 0.10 |
| 188-208h | 0 | 0 | 0 | 0.04 | 0.04 | 0.08 |
| 208-248h | 0 | 0 | 0 | 0.02 | 0.04 | 0.15 |
| 248-288h | 0 | 0 | 0 | 0.03 | 0.02 | 0.02 |
| 288-328h | 0 | 0 | 0 | 0.04 | 0.06 | 0.02 |
| 328h bulk | 0 | 0 | 0 | 0.05 | 0.04 | 0.03 |

N/A: Not Applicable

Log(HPC) (CFU/mL)

| Sample | Run 1 | Run 2 | Run 3 | Run 4 | Run 5 | Run 6 |
|-----------|-------|-------|-------|-------|-------|-------|
| 0-20h | 5.21 | 5.89 | 6.08 | 5.69 | 6.00 | 5.86 |
| 20-40h | 4.98 | 5.84 | 5.92 | 5.70 | 5.83 | 5.63 |
| 40-80h | 4.90 | 5.64 | 5.98 | 5.76 | 5.74 | 5.65 |
| 80-120h | 5.00 | 5.66 | 5.71 | 5.95 | 5.69 | 5.49 |
| 120-160h | 5.08 | 5.70 | 5.84 | 5.90 | 5.86 | 5.58 |
| 160h bulk | 5.11 | 5.81 | 5.72 | 5.69 | 5.76 | 5.52 |
| 160h film | 4.70* | 5.43* | 5.56* | 6.43* | 6.25* | 6.05* |
| 165h bulk | 1.11 | 1.60 | 1.00 | N/A | N/A | N/A |
| 165h film | 4.48* | 5.95* | 3.20* | N/A | N/A | N/A |
| 168-188h | 5.15 | 5.81 | 4.46 | 5.15 | 5.60 | 3.48 |
| 188-208h | 5.20 | 5.61 | 6.07 | 5.42 | 5.81 | 5.53 |
| 208-248h | 5.11 | 5.43 | 6.04 | 5.83 | 5.76 | 5.62 |
| 248-288h | 4.75 | 5.63 | 5.72 | 5.63 | 5.91 | 5.79 |
| 288-328h | 4.86 | 5.49 | 5.67 | 5.63 | 5.69 | 5.76 |
| 328h bulk | 5.00 | 5.57 | 5.68 | 5.57 | 5.76 | 5.85 |
| 328h film | 4.78* | 6.46* | 6.26* | 6.58* | 6.53* | 6.36* |

*: Biofilm samples expressed as CFU per cm².

N/A: Not Applicable

Appendix 3

Oocyst quantification

To perform an approximate calculation of the fate of oocysts in the experimental system, the following “mass balance” has been performed.

Mean input to system: 9.3×10^5

Mean initial outputs from system:

0-160h water 2.9×10^3

160h bulk 3.7×10^3

160h slurry 7.6×10^3

Mean initial retention in system:

160h biofilm 1.1×10^4

Runs 1-3, subsequent output from system:

165h bulk 6.6×10^3

168-328h water 6.5×10^2

328h bulk 5.3×10^2

328h slurry 8.6×10^2

Runs 1-3, final retention in system:

328h biofilm 8.3×10^3

Runs 4-6, subsequent output from system:

168-328h water 4.8×10^2

328h bulk 3.3×10^1

328h slurry 7.5×10^3

Runs 4-6, final retention in system:

328h biofilm 3.9×10^3

Runs 1-3, total output from system:

2.3×10^4 + final retention estimate = 3.1×10^4

Runs 4-6, total output from system:

2.2×10^4 + final retention estimate = 2.6×10^4

