Evaluating the effects of strain selection on the attenuation of *Bacillus subtilis* spores through saturated porous media

by

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Increasingly stringent water quality regulations concerning microbiological parameters govern the use of groundwater resources that are vulnerable to mixing with surface waters. These drinking water sources are at higher risk for infiltration by pathogenic microorganisms, including the oocysts of the human enteroparasite *Cryptosporidium* spp. *Cryptosporidium* can cause severe gastroenteritis in humans, and the characteristics of *Cryptosporidium* oocysts, including low infectious dose, high resistance to inactivation, and long survival in the environment pose a significant risk to public health if present in treated drinking water. *Bacillus subtilis* is widely used as a surrogate for biocolloid transport in saturated porous media, and recognized as a conservative indicator for the transport of *Cryptosporidium parvum* oocysts during filtration. However, no study has directly compared the transport of spores from different strains within the *Bacillus subtilis* species. Strain variability has the potential to impact retention in porous media based on differences in size and electrophoretic mobility.

The transport behaviour of four strains of *Bacillus subtilis* (wild-type and laboratory type, subspecies *subtilis* and subspecies *spizizenii*; 1.9 to 2.9µm diameter) is contrasted in this research to two sizes of fluorescent polystyrene microspheres (1.1µm and 4.5µm diameter) through packed saturated crushed quartz sand. A peristaltic pump was used to introduce (bio)colloids into the duplicate column apparatus at a loading rate of 0.1m/day. (Bio)colloid removal was assessed and compared by constructing breakthrough curves of normalized concentrations and box-and-whisker diagrams of percent removal of *Bacillus subtilis* strains.

Under unfavourable conditions minimal reduction ($<0.22 \log_{10}$) in effluent spore concentration was observed over the column depth of 15cm. In favourable attachment conditions up to 0.69 $\log_{10}$ reduction was observed but the sampling schedule employed was insufficient to clearly identify a pseudo steady-state plateau. An analysis of variance was used to determine the statistical significance of spore strain, subspecies, and type. A significant difference between the four strains was observed at the lower ionic strength, with spore subspecies and type affecting spore removal in unfavourable conditions ($p < 0.05$). Some sensitivity to settling and laboratory storage suggests that standardized sample handling procedures are required. Differences observed here between the strains of *Bacillus subtilis* spores indicate that riverbank filtration performance assessments and drinking water treatment plant process demonstrations may benefit from a recommended strain for use.

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Dedication

For Veena, who saved me, and then said

“Try a New Experiment”

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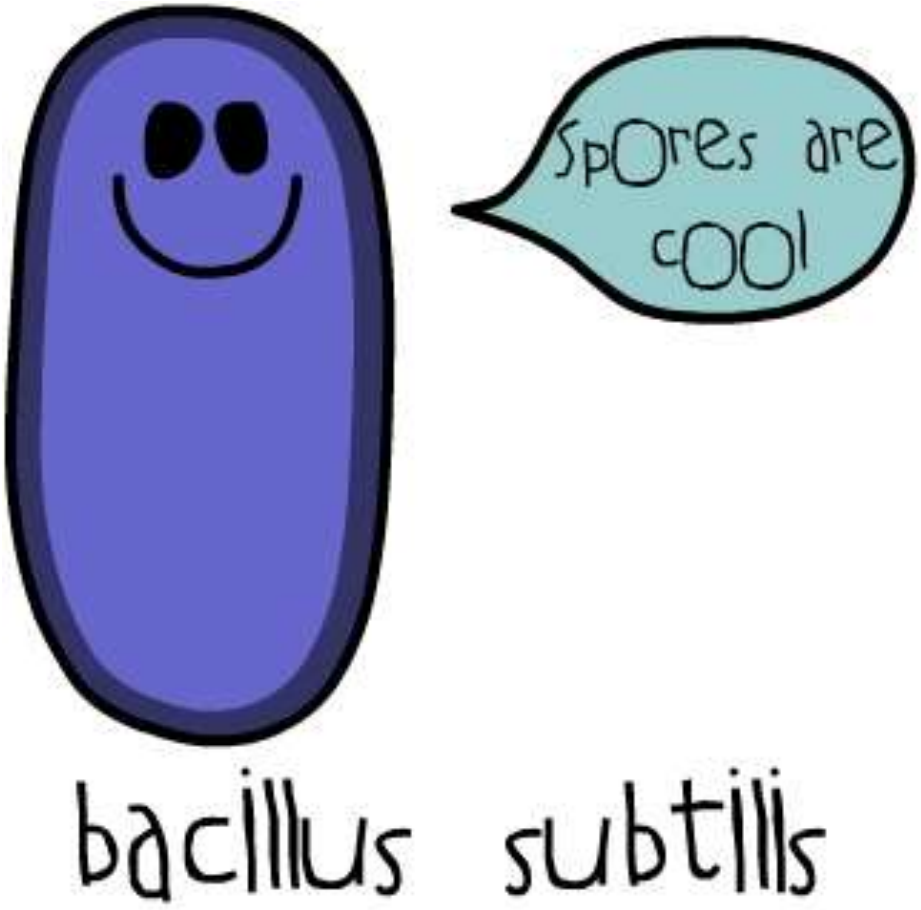
List of Abbreviations

Abbreviation	Description
α	Significance level
AGW	Artificial Groundwater
ANOVA	Analysis Of Variance
BMP	Best Management Practices
BGSC	*Bacillus* Genetic Stock Centre
Br^-	Bromide
BTC	Breakthrough Curve
CDCP	Centre for Disease Control and Prevention
CFU	Colony Forming Units
CFT	Colloid Filtration Theory
DI	Deionized Water
E. coli	*Eschericia coli*
EPA	Environmental Protection Agency
EPS	Extracellular Polymeric Substances
ES	Effective Size
GC	Graduated Cylinder
GS	Grain Size
GW	Groundwater
HEF	High Energy Sonication
ICR	Information Collection Rule
IF	Immunofluorescence
IS	Ionic Strength
KCl	Potassium Chloride
L	Laboratory-Type *Bacillus* strain

Abbreviation	Description
LT1ESWTR	Long Term 1 Enhanced Surface Water Treatment Rule
LT2ESWTR	Long Term 2 Enhanced Surface Water Treatment Rule
MS	MicroSpheres
MOE	Ministry of the Environment
NR	Nile Red
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PV	Pore Volume
RBF	Riverbank Filtration
SD	Standard Deviation
spp.	species pluralis (multiple species)
SPSS	Statistical Product and Service Solutions
SWTR	Surface Water Treatment Rule
TOR	Terms of Reference
UC	Uniformity Coefficient
UV	Ultra-violet
US	United States
WT	Wild-Type *Bacillus* strain
YG	Yellow Green
ZP	Zeta Potential (mV)



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Chapter 1 Introduction

Pathogen Removal During Drinking Water Treatment

Water utilities are responsible for meeting consumer demands for high quality water while following treatment guidelines and adhering to increasingly stringent finished water quality regulations. Drinking water sources include groundwater, surface water, and groundwater under the influence of surface water (commonly referred to as GUDI in Canada and GWUDI in the United States). Source waters vary in quality, but groundwaters may be vulnerable to infiltration of water and wastewater containing any number of industrial, agricultural, pharmaceutical, and chemical contaminants. Contaminants of primary interest are those that threaten public health, and include microbial pathogens such as *Cryptosporidium parvum*.

Granular media filtration is recognized as an important process in the removal of pathogenic (i.e. disease-causing) microorganisms during drinking water treatment. In various forms, including riverbank filtration and engineered granular media, filtration offers a robust solution to minimize the risks and deleterious human health impacts associated with outbreaks of waterborne-disease because it can remove pathogens and/or reduce pathogen loading into subsequent treatment processes (e.g. disinfection) via several mechanisms. Depending on the form of filtration used, these may include physicochemical filtration, aggregation and die-off, and physical straining (Yao et al., 1971; McDowell-Boyer et al., 1986; Ryan and Elimelech, 1996; Ginn et al., 2002; Bradford et al., 2004; Tufenkji and Elimelech, 2004; Foppen et al., 2005; Pang et al., 2005). Riverbank filtration (RBF) is a relatively low cost technology that utilizes naturally occurring saturated porous media (riverbed materials and alluvial sands) to improve water quality by drawing surface water down through bed materials and into extraction wells. Wells are drilled into shallow aquifers adjacent to surface water sources and contaminants are removed or degraded during infiltration, while wells are recharged from the surface waters (Emelko et al., 2010). In North America, RBF and rapid sand filtration are commonly utilized as part of a multi-barrier approach for reducing pathogen risk associated with untreated drinking water, and protecting public health.

Pathogen removal via filtration is described by classic filtration theory as a two step process: first, particles in fluid are transported to the vicinity of the surface of media grains (collectors); second, attachment may occur (McDowell-Boyer et al., 1986; Ryan and Elimelech, 1996). Pathogens and surrogates are considered particles of colloidal size (<10µm) which determines, in conjunction with hydraulic conditions and the physical properties of both colloid and collector, their contact efficiency with collectors (η) and subsequent attachment. Bacterial-sized particles (~1µm) fall at, or near, a minimum colloid contact efficiency so if all other factors are consistent, should be removed to a lesser extent than larger colloids like the oocysts of *Cryptosporidium* spp. (4-6µm). Colloid attachment to surfaces is described by attachment efficiency (α), which is influenced by factors such as electrostatic interactions, colloid-colloid interactions and steric repulsion; changes in pH and ionic strength may cause variations in colloid removal by porous media (Ryan and Elimelech, 1996). Interactions between the colloid and the collector are determined using the DLVO theory (Derjaguin-Landau-Verwey-Overbeek) which combines Lifshitz-Van der Waals interactions with electrostatic double layer interactions to determine if deposition is favourable or unfavourable (Derjaguin and Landau, 1941; Verwey and Overbeek, 1948). Most colloids in natural water, including *Cryptosporidium,* have negatively charged surfaces (zeta potential typically between -5 and -50 mV) so electrostatic repulsion governs attachment at lower ionic strengths (unfavourable conditions for deposition) (Bradford et al., 2007; Bradford et al., 2004; Tufenkji et al., 2004; Dai and Hozalski, 2003; Lytle et al., 2002). Many studies have demonstrated deviations from classic filtration theory due to microbial pathogen or surrogate surface characteristics that theory does not describe, including the production of extracellular polymeric substances or the presence of surface appendages (Castro and Tufenkji, 2008; Tufenkji and Elimelech, 2005). Accordingly, bench-scale assessments of surrogate and/or pathogen removal during filtration through saturated porous media remain the most common approach for optimizing filtration during drinking water treatment and preventing pathogen passage through filtration processes (Emelko et al., 2003).

The presence of pathogenic microorganisms (viral, bacterial, protozoan or parasitic pathogens) in treated drinking water can result in widespread and serious human health impacts. Waterborne disease can cause symptoms ranging from mild gastrointestinal discomfort, to severe and debilitating gastroenteritis, and present a

risk of death in the immunocompromised (Chappell et al., 1996; Karanis et al., 2007; Kraus and Griebler, 2011). In May 2000, in Walkerton, Ontario, an outbreak of the pathogenic bacterium *Eschericia coli* 0157:H7 resulted in illness in 2000 individuals, hospitalization of 65, and the death of seven (O'Connor, 2002). Drinking water sources in Walkerton were contaminated with agricultural runoff and received inadequate treatment prior to distribution, allowing the contagious enterohemorrhagic strain of *E. coli* to infect the population (O'Connor, 2002). Waterborne disease can also be caused by pathogenic protozoa (e.g. *Giardia lamblia*, *Cryptosporidium parvum*), pathogenic parasities (e.g. nematodes) or pathogenic viruses (e.g. Norovirus) and can affect large populations. For example, in 1993 an outbreak in Milwaukee caused approximately 403,000 suspected illnesses, 1000 hospitalizations and 100 deaths (Rose, 1997).

Of the pathogens found in drinking water sources, the human pathogen *Cryptosporidium* spp (*Cryptosporidium parvum* and *Cryptosporidium hominis)* is of significant concern. *Cryptosporidium* is a protozoan parasite that causes gastroenteritis in humans. It is ubiquitous in the natural environment and infectious at very low doses (Peeters et al., 1989; Chappell et al., 1996). It is excreted by infected hosts as environmentally hardy oocysts, which remain viable for months or longer (Lederberg et al., 1992; DuPont et al., 1995; Okhuysen et al., 1999). *Cryptosporidium* oocysts are small (four to six µm) and can cause infection upon ingestion, resulting in symptoms of diarrhea, nausea, vomiting, general malaise, myalgias, and often fever (DuPont et al., 1995; Okhuysen et al., 1999). These symptoms can last up to 30 days in a healthy individual, while immunocompromised persons may be unable to clear the pathogen without medical assistance (Lederberg et al., 1992). The illness can be quite severe. Although reporting underestimates the actual number of cases, *Cryptosporidium* spp. affects thousands of individuals annually in the United States alone; in the most recent Surveillance Summary (2010) the United States Centre for Disease Control and Prevention (CDCP) reported 6479, 11657, and 10500 cases of individual Cryptosporidiosis in the United States. 2006, 2007, and 2008 respectively. *Cryptosporidium* spp. is also particularly resistant to chemical disinfectants commonly used in drinking water treatment; chlorination and chloramination are ineffective at inactivating *Cryptosporidium* spp. oocysts, while ozone dose requirements for

inactivation may be economically infeasible (Peeters et al., 1989; Fricker and Crabb, 1998; Shammas and Wang, 2011). Collectively, the characteristics of *Cryptosporidium* spp. oocysts, including low infectious dose, high resistance to inactivation, small size, frequency of occurrence, and long survival in the environment indicate that it poses a significant risk to public health.

Drinking water treatment performance demonstrations can be used to indicate microorganism reductions through treatment processes (Yates et al., 1997; Baudin et al., 1999; Brown and Cornwell, 2007). Some metrics of water quality, such as turbidity or total aerobic spore counts may be used in demonstrations to indicate the level of treatment or removal of a target compound that is achievable using a certain process. Changes in parameters such as turbidity are useful for general performance monitoring, but are not well correlated with actual microbe reductions (Payment and Locas, 2011; Ahmad et al., 2009; Mons et al., 2009; Cizek et al., 2008; Huck et al., 2004; Smith and Perdek, 2004; Nieminski, 2003). Surrogates may correlate somewhat with microbe transport and behavior; they are assumed to behave similarly to pathogenic microorganisms-of-interest and therefore they are used to provide pseudo-quantitative data on pathogen removal and treatment. Surrogates of colloidal size (<10microns) are often used to study pathogen attenuation through saturated porous media (Knappett et al., 2008; Passmore et al., 2010). Several surrogate organisms have been proposed to assist in estimating the transport and retention of *C. parvum* in porous media during filtration including polystyrene microspheres and aerobic and anaerobic endospores (USEPA 2006).

During treatment plant performance demonstrations, monitoring the passage of naturally occurring total aerobic spores (TAS) through treatment processes has been suggested to provide a conservative estimate of *Cryptosporidium* spp. removal potential; this method is limited if spore concentrations are not high ($<1.0 \times 10^4$ spores/L) (Brown and Cornwell, 2007). To reach adequate spore concentrations, some studies have introduced high-concentrations of spores into wells subject to riverbank filtration or into drinking water treatment plant processes and monitored spore concentration reductions over distance travelled or after individual treatment processes (Pang and Close, 2005; Huck et al., 2002; Nieminski and Bellamy, 1998; Pang et al., 1998). *Bacillus subtilis*, an aerobic bacterium capable of forming environmentally

resilient endospores, has also been suggested as a surrogate for use in RBF and other filtration demonstrations. However, the strain suggested for use when spores are introduced into the environment under investigation is not specified, nor are spore source, propagation, or purification protocol. There are over 50 strains of *Bacillus subtilis* available from the Bacillus Genetic Stock Centre (BGSC). The well-characterized laboratory strains (lab-type) are easy to cultivate and homogeneous, while the more recently-isolated strains sourced from the natural environment (wild-type) retain the ability to form complex growth structures, have increased motility and show variable surface characteristics (Rose et al., 2007). Accordingly, if *Bacillus* spores are to be introduced into treatment streams or used to evaluate process performance, the impact of strain selection on spore fate and transport in the treatment process must be understood.

1.1 Research Scope and Hypothesis

The scope of this research is to compare the removal of spores from different strains of *Bacillus subtilis* during passage through saturated porous media. Lab-type strains differ from environmental strains in their production of extracellular polymeric substances, swarming mobility and formation of complex architectures which may impact their removal during filtration. Four strains of *Bacillus subtilis* were propagated for use in the present investigation. These included two well-studied domesticated strains (BGSC 2A9 / W23, and BGSC 1A1 / 168) and two wild-type strains (ATCC 6633, NCIB 3610). Each strain was cultivated according to standard methods. Sporulation was stimulated by the nutrient exhaustion approach (Nicholson and Setlow, 1990). Spores were suspended in artificial groundwater (0.1mM and 10mM KCl) and introduced into saturated quartz media in bench-scale filtration columns operated in a down-flow mode at approximately 1.2 mL/min (0.1m/hour). The effluent breakthrough curves of wild-type and laboratory strains of *Bacillus subtilis* spores were then evaluated and compared. Due to the ability of wild-type strains to produce larger amounts of extracellular polymeric substances, potentially reducing spore transport, it is expected that wild-type strains ATCC 6633 and NCIB 3610 will demonstrate greater mean $\log_{10}$ removals during transport through saturated quartz media.

1.2 Research Objectives

The primary goal of this thesis work was to provide information on the transport of laboratory and wild-type strains of *Bacillus subtilis* during granular media filtration. Specifically the objectives of this research were to:

1. Develop the skills and knowledge necessary for the propagation, purification, and enumeration of four strains of *Bacillus subtilis* spores;
2. Evaluate the relative impact of spore strain selection on their transport through saturated porous media under conditions representative of riverbank filtration
3. Measure the electrophoretic mobility (representaed as zeta potential) of domesticated and wild-type strains in the selected AGW matrices;
4. Contrast spore transport to the transport of bacteria-sized and protozoan-sized microspheres (1.1µm and 4.5µm, respectively) as surrogates with relatively inactive surfaces; and
5. Provide recommendations for spore suspension use to minimize sources of uncertainty and improve the comparability of experimental outcomes from laboratory and field investigations of spore transport through saturated porous media.

1.3 Thesis Organization

Chapter Two presents background information on *Cryptosporidium parvum* and its potential surrogates, as well as the regulatory context for the use of aerobic spores in treatment process performance demonstrations and colloid filtration. Chapter Three provides a detailed description of the laboratory methodology and research approach used in this study. Experimental results and discussion are presented in Chapter Four. Chapter Five contains conclusions and recommendations for future work and use of *Bacillus subtilis* spores.

Chapter 2 Literature Review

2.0 Introduction

Pathogen survival in the subsurface and transport through saturated porous media must be better understood to improve removal during natural and engineered drinking water treatment. In particular, the oocysts of the parasite *Cryptosporidium* present a treatment challenge as they are widespread in surface waters, can have serious human health consequences at low doses, and are resistant to conventional chemical disinfection. High analytical costs and lengthy assay times preclude direct monitoring of oocyst concentrations through drinking water treatment processes. Indirect water quality parameters, indicators, and surrogates have been proposed to provide pseudo-quantitative data on the behaviour of *Cryptosporidium* during treatment via many processes including porous media filtration. The aerobic endospores of the common soil bacterium *Bacillus subtilis* have been suggested as conservative estimators of the transport of human pathogen *Cryptosporidium* spp (*Cryprosporidium parvum* and *Cryptosporidium hominis,* referred to as *Cryptosporidium* herein) oocyst transport and retention during filtration. Strain variability may impact usability as a surrogate in microbial risk assessments, performance demonstrations of treatment plants and analyses of riverbank filtration; this thesis addresses the impact of strain variability on *Bacillus subtilis* spore transport in saturated sand.

2.1 *Cryptosporidium parvum*

2.1.1 Health Effects

The obligate protozoan entereoparasites of the genera *Cryptosporidium* remain of concern during drinking water treatment. Between 1983 and 2002, *Cryptosporidium* species accounted for 50.8% of all reported worldwide outbreaks of water-associated disease attributable to protozoan parasites (165 of the 325 disease outbreaks) (Karanis et al., 2007). *Cryptosporidium* is ubiquitous in the environment (Ongerth and Stibs, 1987; LeChevallier et al., 1991; Carmena et al., 2007) and persists for several months or more in a viable, infectious form (Montemayor et al., 2005; Karanis et al., 2007). It is resistant to routine chemical disinfection and presents analytical challenges, requiring large sample volumes due to low environmental concentrations (reported from <2 to >18 oocysts/litre in surface water bodies) (Ongerth and Stibs, 1987; Payment and

Franco, 1993; Baudin and Laîné, 1998; Carmena et al., 2007; Ryu and Abbaszadegan, 2008; Ahmad et al., 2009).

Cryptosporidium can contaminate drinking water sources, including groundwater, via infiltration from surface waters that have received inadequately treated municipal wastewaters, stormwater overflows, or agricultural runoff (Ongerth and Stibs, 1987; LeChevallier et al., 1991; Smith et al., 1995; Solo-Gabriele and Neumeister, 1996; Hancock et al., 1998; Stott et al., 2003). *Cryptosporidium* infects numerous mammalian hosts, including waterfowl, and completes its life cycle within a single host resulting in the excretion of large numbers of environmentally robust and small, spherical oocysts (four to six µm in diameter) (Peeters et al., 1989; Smith et al., 1995; Smith and Lloyd, 1997; Schijven and Hassanizavadeh, 2000; Pang et al., 2005). The ingestion of as few as ten oocysts (some suggest one) can result in infection (DuPont et al., 1995; Okhuysen et al., 1999; Smith and Perdek, 2004). An individual suffering from Cryptosporidiosis may excrete 10^8 to 10^{10} oocysts per bowel movement for up to two months after clinical symptoms have ceased (Jokipii and Jokipii, 1986; Chappell et al., 1996; Smith et al., 1995). Once excreted, oocysts can survive for months suspended in water, presenting a threat to drinking water sources (Montemayor et al., 2005; Karanis et al., 2007).

The illness, Cryptosporidiosis, is a parasitic infection affecting the epithelial cells of the gastrointestinal, biliary, and respiratory tract of humans. Symptoms can persist up to 30 days in healthy individuals and include diarrhea, nausea, vomiting and general malaise, myalgias, and often fever (Lederberg et al., 1992). In immunocompetent individuals the infection is self-limiting; immunocompromised individuals, including young children and the elderly, may be unable to clear the parasite on their own, with symptoms possibly developing into life-threatening infections or leading to cholangitis or pancreatitis (Hunter and Nichols, 2002). The infection is identified using fecal smears or biopsies and should be detectable immediately upon infection. Cryptosporidiosis is transmitted via the fecal-oral route; without proper hygiene an individual is liable to reinfect themselves or their close community via contact with infected environmental surfaces, or improperly handled food and water (Lederberg et al., 1992; Juranek, 1995). Contamination of community water systems can quickly place large numbers of consumers at risk (Fox and Lytle, 1996; Karanis et al., 2007). In

Spain, Carmena et al. (2007), found that 30.8% of the treated water samples collected from small treatment facilities, and 26.8% of tap water in municipalities with only chlorination treatment contained *Cryptosporidium* oocsyts. In the United Kingdom, legislation set a legal limit of 1 oocyst/10L of water as a standard for water treatment but *Cryptosporidium* can pose health risks even at this dose (as cited in Barbosa et al., 2008; Shammas and Wang, 2011). With such a low infectious dose, the risk presented by oocyst presence in drinking water supplies creates a significant concern (LeChevallier et al., 1991).

2.1.2 Outbreaks

The first documented outbreaks of *Cryptosporidium*-associated waterborne disease occurred in the early 1980s. Initially the disease was only associated with immunocompromised individuals, but as laboratory diagnostic techniques improved, cases were diagnosed in immunocompetent individuals (CDC, 1995). Early outbreaks were small (only three outbreaks affecting over 2,000 people) and little was known about the pathogen at the time (Solo-Gabriele and Neumeister, 1996). In March of 1993, a large outbreak was associated with the municipal drinking water system in Kitchener-Waterloo, Ontario and caused illness in approximately 23,900 people (Craun et al., 1998). Only a few months later, the United States had the largest outbreak of a waterborne disease in the history of the country, affecting 403,000 individuals in Milwaukee, Wisconsin (MacKenzie et al., 1994). Reviews of the circumstances leading up to the outbreaks suggest that both were caused by inadequately addressing changing source water conditions that compromised treatment capacity. Despite meeting treatment requirements, it has been suggested that heavy snowmelt and spring runoff caused surface water to be contaminated by runoff from agricultural activity in the Grand River watershed in Ontario, which compromised the previously unproblematic well water (Craun et al., 1998). In Milwaukee, inadequate oocyst removal by the coagulation and filtration processes occurred when spring rains caused increased turbidity and bacterial counts in the source water drawn from Lake Michigan (MacKenzie et al., 1994). Human sewage, direct deposition from cattle along tributaries, and wastes received from slaughterhouses were named as potential oocyst sources in the Milwaukee Cryptosporidiosis outbreak. The severity of these outbreaks highlighted the risks of contamination of drinking water supplies with as yet

unregulated pathogenic microorganisms (MacKenzie et al., 1994; Fricker and Crabb, 1998).

The Milwaukee outbreak potentially caused the deaths of 112 individuals, and drew the focus of water providers and regulators to the presence of pathogenic *Cryptosporidium* in treated drinking water. An estimated 300,000 cases of Cryptosporidiosis occur each year in the United States and are caused by consumption of contaminated food or water or participating in recreational water activities and accidentally ingesting contaminated waters (Mead et al., 1999). Only a small fraction of cases are reported and confirmed with laboratory analysis (11,657 and 10,500 cases in 2007 and 2008, respectively) (CDCP, 2010). The reasons for underreporting include asymptomatic infected individuals, infrequent laboratory examination of non-bloody diarrheal diseases, infrequent testing for *Cryptosporidium* specifically, and incomplete reporting even in cases with positive laboratory results (Mead et al., 1999; Berger and Weintraub, 2005; Jones et al., 2004). Recurring outbreaks around the world, some associated with contaminated recreational waters and some with municipally-distributed drinking water, continue to drive research addressing the removal of *Cryptosporidium* during drinking water treatment (Karanis et al., 2007).

2.1.3 Disinfection

The outbreaks of cryptosporidiosis in the early 1980s caused alarm largely because the relatively unknown etiological agent was penetrating drinking water treatment systems and surviving disinfection (Karanis et al., 2007; Smith and Lloyd 1997). *Cryptosporidium* oocysts are resistant to common chemical disinfectants used in drinking water treatment: free chlorine and monocloramines, the two most commonly used, are essentially ineffective at the dosages and contact times currently in practice (Korich et al., 1990; Chauret et al., 2001; Verhille et al., 2003). This resilience is associated with the rigid, double cell wall in oocysts that are shed from the host into the environment (Templeton et al., 2004). Oocyst inactivation required 90 minutes of exposure to 80ppm of chlorine or monochloramine to achieve 90% inactivation according to Korich et al. (1990). With the possible exception of ozone, the use of rapid filtration or chlorination alone should not be expected to remove and inactivate *Cryptosporidium* oocysts to the levels required for safe drinking water (Carmena et al., 2006). Unfortunately, the prohibitive costs of ozonation leave many utilities reliant

exclusively upon filtration for oocyst concentration reductions (Peeters et al., 1989; Fricker and Crabb, 1998; Shammas and Wang, 2011). Accordingly, the regulations outlined by the USEPA acknowledge that *Cryptosporidium* control relies on physical removal via physico-chemical filtration of oocysts to acceptably low concentrations (USEPA, 1998; USEPA, 2006).

2.1.4 *Oocyst* Removal

Cryptosporidium removal from raw source waters is typically achieved using conventional treatment processes including: coagulation-flocculation, sedimentation, and granular-media filtration, which are followed by disinfection (chlorine, chloramines, ozone, or UV irradiation). Oocyst removals by coagulation, flocculation, or sedimentation range from 0.5-2.0 log (Emelko et al., 2005; Dugan et al., 2001; Emelko, 2001; Baudin and Laîné, 1998). Oocyst removal is dependent upon the site-specific water quality and filter operational conditions. Huck et al. (2002), evaluated oocyst removal throughout filter operation at two plants, finding a reproducible minimum of 2-log removal at optimal conditions. Oocyst removal capacity deteriorated at the end of the filter run in one plant and if suboptimal coagulation occurred. The expense of conventional technologies leaves some utilities looking for alternative treatment methods. Riverbank filtration is a low-cost pre-treatment option, anticipated to improve water quality through subsurface filtration. Riverbed and aquifer materials, composed of sands (at least 10% with grain size <1.0mm), are capable of pathogen removal via a number of processes (inactivation, predation, straining, physicochemical attachment) (Schijven et al., 2000, 2003a, 2003b). It is difficult for regulatory bodies to prescribe treatment credits for riverbank filtration systems without a thorough site-specific assessment of pathogen removal capacity.

2.1.5 Regulations

The United States (US) Surface Water Treatment Rule (SWTR) was finalized in 1989 to address the increasing problem of waterborne disease outbreaks with a focus on *Giardia lamblia* and enteric viruses. This rule requires filtration and disinfection of all surface water supplies and offers filtration avoidance criteria (USEPA, 1989). The aim of the SWTR was to reduce the risk of acquiring an infection of *Giardia lamblia* due to waterborne transmission to below 10^{-4} per person per annum, based on an acceptable infection rate of one microbiologically-caused illness per year per 10,000 people (USEPA 1989; Regli et al., 1991). Drinking water treatment plants were required to

design processes and operate to achieve at least 99.9% removal (3-log) and/or inactivation of *Giardia* cysts. This calculation assumed an average of 7 cysts per 100 litres in raw water, with specifications for greater treatment in situations of poor water quality (LeChevallier et al., 1991).

By the time of the Milwaukee outbreak of cryptosporidiosis in 1993, *Cryptosporidium parvum* was recognized as an agent of waterborne disease and was on the USEPA's priority list of substances requiring regulation (USEPA, 1991; Craun et al., 1998). By 1996, it was recognized that conventional treatment processes operating at minimum compliance levels could be ineffective at preventing the transmission of *Cryptosporidium* through to treated drinking water (Solo-Gabriele and Neumeister, 1996). Consequently, in 1996, the Information Collection Rule (ICR) required large utilities to monitor surface waters for *Cryptosporidium* and *Giardia*, along with enteric viruses (USEPA, 1996) to support and inform the development of the Interim Enhanced Surface Water Treatment Rule (IESWTR) (amendments to the SWTR), which addressed *Cryptosporidium* treatment. The IESWTR required a minimum 2-log (99%) reduction in *Cryptosporidium* by treatment, for systems serving over 10,000 people (USEPA, 1998).

More recently, the Long Term 1 and 2 Enhanced Surface Water Treatment Rules (LT1ESWTR and LT2ESTWR) addressed the passage of microbial contaminants, and specifically *Cryptosporidium*, into drinking water by requiring system owners to select treatment techniques to adequately address their site-specific needs as outlined by the 'microbial toolbox' (USEPA 1998; USEPA 2006). The objective of the LT2ESWTR is to reduce the incidence of pathogen-associated disease and applies to all community and non-community water systems that use surface water or ground-waters under the influence of surface waters (GWUDI). These systems are required to comply with potentially increased *Cryptosporidium* treatment requirements; as source-water concentrations of *Cryptosporidium* increase, treatment requirements also increase (USEPA, 2001). The risk-based treatment requirements assign a bin classification following a source water monitoring program (for systems serving >10,000 people). For example, systems with >3 oocysts/L (Bin 4), require an additional 2.5 to 3-log treatment, one of which must be achieved using ozone, chlorine dioxide, UV, membranes, bag/cartridge filters, or bank filtration. Unfiltered systems are required to disinfect using two of the three methods proven to be effective at *Cryptosporidium*

inactivation (ozone, ultraviolet light (UV), or chlorine dioxide). Filtered systems using alternative filtration technologies (excluding direct, slow sand, diatomaceous earth filtration or conventional filtration with softening) may require at least 5.5 log inactivation/removal of *Cryptosporidium* (USEPA, 2003).

To achieve the required *Cryptosporidium* removals, US utilities need the ability to quantify *Cryptosporidium* concentrations in source water and after treatment (Shammas and Wang, 2011). The US LT2ESWTR allows removal credits for riverbank filtration at sites where aquifer materials are primarily sandy and extraction wells are set back approximately 7.5 meters from the surface water source. Such wells receive 0.5-log removal/treatment credit, while wells with setback distances of 50 feet (15.25m) receive 1.0-log treatment credit. To receive higher removal credits, utilities must conduct performance evaluations using an approved challenge particulate (USEPA, 2003).

According to the Ontario Ministry of the Environment (MOE), well water is considered ground water under the direct influence of surface water (GUDI in Canada, referred to as GWUDI herein) where it contains surface water organisms, including *Cryptosporidium* (MOE, 2001). Ontario drinking water standards require chemically assisted filtration and disinfection or equivalent treatment unless a utility can demonstrate effective *in situ* filtration. Utilities interested in demonstrating *in situ* filtration under Ontario's Ministry of the Environment terms of reference (TOR), must submit a report document that includes a thorough characterization of the hydrogeological setting, an evaluation of the source water quality including seasonal variations and stormwater effects, and a determination of filtration requirements (MOE, 2001). The raw water must be of 'good microbiological quality' to be considered effectively filtered by the *in situ* riverbank materials. At present, to determine raw water quality, utilizes must conduct a microbiological water quality and particle count assessment as well as a hydrogeological evaluation to assess *Cryptosporidium* oocyst removal capacity.

2.1.6 Analytical Challenges

Cryptosporidium oocysts present unique analytical challenges. First, low occurrence in the environment combined with low detection limits (one oocyst per filter) requires the transportation and concentration of large-volume samples when on-site sample

concentration is not possible (Howe et al., 2002; Nieminski, 2003; Verhille et al., 2003; Montemayor et al., 2005). Sample volume requirements vary according to source water quality and oocyst concentrations, but need to meet detection limits. Poor source water quality (e.g. high turbidity) may interfere with oocyst detection. Second, the detection of oocysts by fluorescence microscopy of stained samples (USEPA method 1623) is dependent on the expertise of laboratory personnel (USEPA, 1999; Hsu et al., 2002; Carmena et al., 2006). Microbiological assays via microscopic observation for *Cryptosporidium* are time consuming, requiring staining and oocyst recognition; staining may be nonspecific making oocyst recognition dependent on personnel experience and expertise (Keswick et al., 1982; Weitzel et al., 2006; Ahmad et al., 2009).

Quantification of oocysts in collected samples is also dependent upon the methods used for collection and analysis (Butler and Mayfield, 1996; Howe et al., 2002; Montemayor et al., 2005; Ryu and Abbaszadegan, 2008). Analyses can be costly: the filters required for *Cryptosporidium* analysis cost approximately $100 per filter and a further $500 per sample for analysis when samples are sent to an accredited laboratory. The LT2ESTWR acknowledged the expense of *Cryptosporidium* analyses: filtered systems serving fewer than 10,000 were required to conduct one year of monitoring for *Escherichia coli* (*E. coli*) and *Cryptosporidium* monitoring was triggered only if *E. coli* levels were greater than 10CFU/100mL for lakes or greater than 50CFU/100mL for streams (USEPA, 2003). Other *Cryptosporidium* detection methods are available (PCR, flow cytometry) and may be less expensive and more efficient at recovery (30 to 80% recovery compared with of 20 to 60% of Method 1623), but require low turbidity samples, expensive laboratory equipment, and cannot determine oocyst viability (PCR) (Montemayor et al., 2007; Barbosa et al., 2008; Emelko et al., 2008; Ryu and Abbaszadegan, 2008). In their assessment of a new flow cytometry method, Barbosa et al. (2008) estimated the per sample cost of consumables to be 2.5 Euros, once the required laboratory equipment was in place. Using specific, labeled antibodies, they were able to clearly distinguish fluorescing oocysts from debris and other microorganisms in the samples.

Although oocysts are commercially available for laboratory investigations, Chauret et al. (2001), observed differential resistance to chemical disinfection based on source of the oocyst. Oocysts ordered from three different laboratories but processed using

identical protocols were exposed to chlorine dioxide, with k (pseudo-first-order reaction rate constant) values of -0.00233, -0.0035 and -0.0270 liters per mg-min calculated from a most probable number cell culture assay for the three sources of oocysts. Dai and Hozalski (2003) found the size of oocysts from two sources differed by 0.5µm, while Kuznar and Elimelech (2004), found different ranges in zeta potential between two batches of oocysts. Nieminski et al. (2010), found different cell antibody stains used for analysis of *Cryptosporidium* concentrations showed variability, with one stain consistently producing higher concentration values. These results highlight some of the uncertainty in *Cryptosporidium* enumeration.

Analytical challenges, in conjunction with the pressing need for information concerning the transport of *Cryptosporidium* through filtration processes, have led to the use of surrogates for investigations of *Cryptosporidium* transport through various media. Monitoring of filtration performance offers the best option for the control of *Cryptosporidium* in water treatment systems and surrogates may offer assistance by providing pseudo-quantitative data to better enable assessment of filter or RBF performance (Smith and Lloyd, 1997; Craun et al., 1998).

2.2 Surrogates

Although the most effective and insightful method of pathogen monitoring would be the presence or absence of the microorganism itself, for *Cryptosporidium*, costly analyses, result variability (method and stain biases), cumbersome concentration and identification methods and lengthy processing times have necessitated reliance on indicators and/or surrogates during drinking water treatment performance assessments (Brown and Cornwell, 2007; Payment and Locas, 2011). The idea of replacing organisms that present analytical challenges with other organisms is not new: in 1978, Wood and Ehrlich selected baker's yeast (*Saccharomyces cerevisiae*) as a microbial tracer during field investigations of bacterial movement in groundwater. They selected *Saccharomyces cerevisiae* because it was readily available, easily analyzed, and thought to be reasonably similar in surface properties to bacteria (Wood and Erhlich, 1978; McDowell-Boyer et al., 1986).

Today, organisms that are suitably similar to the pathogenic microorganism-of-interest are selected for use in situations where using the pathogen itself is impractical. Similarities are based upon the area of investigation (ie. what the surrogate is being

used to study) and may include size, surface characteristics, chemical resistance, transport behaviour or electrophoretic mobility. Typically, pathogenic microorganisms cannot be used in riverbank filtration performance assessments, so non-pathogenic organisms must act as surrogates to earn removal credits. When using surrogates, it is beneficial to first assess the pathogen itself to ensure the surrogate is an adequate representative (Smith and Perdek, 2004). *Cryptosporidium* surrogates can sometimes provide pseudo-quantitative data on pathogen behaviour, but must be carefully selected and validated. Potential surrogates for *Cryptosporidium* include non-biological metrics of water quality, non-pathogenic microbes or inactivated pathogens, or (bio)colloids including microspheres (Emelko et al., 2004; Emelko et al., 2005; Brown and Cornwell, 2007).

In an assessment of conventional treatment, Dugan et al. (2001), used turbidity, total particle counts, and aerobic endospores as indicators of *Cryptosporidium* oocyst removal during fourteen pilot-scale experiments. The calculated removals of the indicators used were consistently lower than the removal of oocysts, providing conservative removal estimates, which is in agreement with a review conducted by Emelko et al. (2005). They suggested that although endospore removal was not always lower than that of *Cryptosporidium*, it was generally found to be a conservative estimator. Dugan et al. (2001), concluded that the water quality parameters investigated could provide a useful indication of minimum treatment potential during conventional treatment with optimal coagulation. Turbidity or particle counts can also be used during drinking water treatment to indicate a certain level of treatment or clarification has been achieved (Verhille et al., 2003). In their assessment of the efficiency of three treatment plants, LeChevallier and Norton (1992), found that for every log reduction in particle counts (>5µm), there was an associated 0.66 log reduction in the occurrence of *Cryptosporidium*, while for every log reduction in turbidity there was an associated 0.89 log reduction. They highlighted that these significant correlations between indicator and pathogen removal could be site specific and pathogen concentrations should be investigated at each plant. Although fecal indicators, turbidity, and particle counts are more easily measured during treatment and may be used as an index of water quality, other studies, summaries and reviews conclude that they fail to correlate well with pathogen concentrations (Payment and Locas, 2011; Tufenkji and Emelko, 2011, Ahmad et al., 2009; Mons et al., 2009; Cizek et

al., 2008; Ryu and Abbaszadegan, 2008; Emelko et al., 2005; Smith and Perdek, 2004; Nieminski, 2003;).

In the LT2ESWTR, the USEPA defines a surrogate for use in treatment performance demonstrations as a "challenge particulate" that is expected to behave and respond to treatment processes in a fashion similar to the organism-of-interest (USEPA, 2006). A distinction between indicator and surrogate is presented by Tufenkji and Emelko, (2011), where:

> an indicator suggests the presence of a type of contamination because they are often associated with the same source of contamination as the pathogen of interest. Surrogates provide a (pseudo)quantitative assessment of the degree of contamination, that is, the fate and transport of the contaminant(s) of interest in specific environments.

Here, further discussion focuses on the use of surrogates (challenge particulates) to investigate the fate and transport of *Cryptosporidium* in saturated porous media. Under the LT2ESWTR, removal assessments to earn credits towards treatment requirements must be conducted using inactivated *Cryptosporidium* oocysts or a surrogate that has been determined to be removed no more efficiently than *Cryptosporidium* oocysts. This excludes the commonly used water quality indicators turbidity, particle counts and conductivity. The LT2ESWTR further specifies that the concentration of the surrogate must be determined using a method capable of discrete quantification of the specific surrogate (Shammas and Wang, 2011; USEPA, 2006).

Collectively, the literature suggests that ideal surrogates should meet several criteria:

Table 2.1 Suggested Characteristics of an Ideal *Cryptosporidium* Surrogate for Transport Assessments

Surrogates should be:

- Non-pathogenic to humans
- Absent from the water source under examination, or easily distinguishable from any naturally occurring organisms or surrogates used concurrently
- Stable and inert in the environment for the duration of the study period
- Detectable at low concentrations with reliable reproducibility
- Simple to measure
- Easy to use at concentrations allowing log removal estimates

Surrogates should emulate the pathogen in

- detection method
- chemically resistance (or slightly more-so)
- transport behaviour
- presence or concentration in source waters

Adapted from: Keswick et al., 1982; Medema et al., 2003; Nieminski, 2003; USEPA, 2006; Payment and Locas, 2011

These suggested requirements depend primarily on how the surrogate will be used; for example, to study inactivation, to estimate transport behaviour, or to estimate pathogen occurrence in the environment. Once the objective of a study is determined, the surrogate should be carefully assessed. This may include characterization of (bio)colloid surface properties, surrogate response to experimental solution chemistry, detection methods and analytical requirements. Cell size, surface charge (measured as zeta potential), extracellular polymeric substances, appendages, and hydrophobicity should be similar to that of the pathogen represented, or the observed and/or measured response or behaviour should be analogous to that of the pathogen despite differences in those parameters (Keswick et al., 1982; Dai and Hozalski, 2003; Tufenkji and Emelko, 2011). The impact of these characteristics on surrogate removal via filtration will be discussed below (Section 2.4). Ideally, surrogates should assist in determining pathogen-associated risks (Schinner et al., 2010; Schijven et al., 2003a; 2003b).

2.3 *Bacillus subtilis*

The bacterium *Bacillus subtilis* satisfies many of the criteria for an ideal surrogate as listed above. In brief: it is non-pathogenic to humans, its spores are stable and inert under typical conditions of investigation which may include riverbank filtration

performance demonstrations (GW ~4-10°C) and plant performance assessments (GW or GWUDI), it may be present in surface water sources at concentrations up to 10^8 CFU per litre but it can also be easily propagated for investigations involving spiking (ie. the injection of high concentrations of spores to monitor removal capacity), it is easily detectable at low concentrations using a variety of methods (spread plating and filter concentration) and the enumeration methods are relatively easy to learn, requiring minimal expertise (Nieminski and Bellamy, 1998). The detection methods used for *Bacillus subtilis* differ from those used for *Cryptosporidium*. Aerobic endospores of *Bacillus* are also more susceptible to disinfection via chlorine dioxide than the pathogenic oocysts, making them an inappropriate surrogate for disinfection studies (Mamane et al., 2009; Verhille et al., 2003).

The USEPA has endorsed the use of endospores as surrogates of *Cryptosporidium* oocyst transport investigation under the LT2ESWTR (USEPA, 2003). In a review of studies published prior to 2003, the Federal Register highlighted two potential *Cryptosporidium* surrogates for use in $\log_{10}$ removal calculations during transport investigations. Two-non pathogenic organisms, the anaerobic spores of *Clostridia* and aerobic endospores, were sufficiently resistant to inactivation in the subsurface, of similar size and shape to oocysts and ubiquitous in both surface and groundwater (USEPA, 2003). Of the two, aerobic endospores (0.5x1.0x2.0µm in diameter) are closer to the size range of *Cryptosporidium* (4-6µm) in comparison with the smaller (0.3-0.4µm) anaerobic spores. The LT2ESWTR regulation cites several studies that successfully monitored the removal of aerobic *Bacillus* spores over distance to quantify riverbank filtration capacity (Pang et al., 1998; Schijven et al., 1998; Medema et al., 2000; Wang et al., 2001). Riverbank filtration typically occurs in shallow, well-aerated aquifers so *Bacillus subtilis*, as an aerobic spore-former, is suggested as a more suitable surrogate measure for *Cryptosporidium* removal by bank filtration (USEPA, 2003).

Commonly found in soil and surface water, *Bacillus subtilis* is a rod-shaped, gram-positive bacterium with the ability to form an internal endospore (Table 2.2) (Madigan and Martinko, 2005; Chen et al., 2010). These multilayered structures are produced by some bacterial cells in response to stress conditions including reduced availability of essential nutrients such as calcium, nitrogen, or phosphorus (Nicholson and Setlow, 1990; Nicholson et al., 2000). As spores, they are metabolically inactive and unable to

reproduce. Endospores (spores herein) are resistant to ultraviolet radiation, desiccation, high temperatures (≤ 80°C), extreme freezing and chemical disinfectants, including chlorines, for extended periods of time (Nicholson et al., 2000; Chen et al., 2010; Faille et al., 2010). Anti-bacterial agents, including alcohols, household cleaners and detergents that are capable of destroying vegetative cell walls are ineffective against double-layered endospores. This longevity and increased resilience and survival make spores a potential surrogate for encysted parasites, which includes *Cryptosporidium* spp. *Bacillus subtilis* spores have been used as *Cryptosporidium* surrogates in disinfection evaluations (Setlow, 2006; Muhammad et al., 2009), riverbank filtration studies (Pang et al., 2002; 2005; Weiss et al., 2005), fate and transport studies in porous media (Ahimou et al., 2001; Naclerio et al., 2009), and filter-capacity assessments (Yates et al., 1997; Fox et al., 1998; Coffey et al., 1999; Dugan et al., 1999; Chauret et al., 2001; Huck et al., 2001; Heurtas et al., 2003; Verhille et al., 2003; Brown and Cornwell, 2007; Shammas and Wang, 2011).

2.3.1 Strain Variability

Discovered in 1878, aerobic spore-forming bacteria were first described in the literature as *Bacillus globigii* and most likely isolated from a soil sample (Fritze and Pukall, 2001). In 1912 it was named *Bacillus* (BGSC 168) and over 1201 mutants have been derived from this original 'Marburg' strain (Conn, 1930). There are over 48 other *Bacillus* strains that have descended from the original isolates; they are widely used in enzyme production and food quality management research. Many of the commonly-used laboratory strains have adapted to the laboratory environment. These laboratory mutants (laboratory-type, LT herein) are easily cultivated and grow reliably and predictably under documented growth conditions. They differ from naturally occurring, environmental isolates of *Bacillus* which maintain the ability to form fruiting bodies and display complex growth architectures, often behaving as a multicellular organism (Kearns and Losick, 2003; Aguilar et al., 2007). They retain natural genetic competences (Nicholson and Setlow, 1990; Rose et al., 2007) in comparison with LT strains which have lost the ability to swarm on growth agar.

Among the *Bacillus* strains, strain 168 is the most studied (Zeigler, 2013; personal communication). Strain 3610 is considered the "wild" parent of strain 168 and descends from the original Marburg strains (WT). Strain 3610 produces biofilms and

structured colonies, whereas strain 168 does not. During experimentation in the 1940s, strain 168 mutated from 3610 after exposure to UV light.

Strain ATCC 6633 is also a mutant derived during experiments run by Paul Burkholder and known as a Burkholder mutant. In a similar relationship as that of strain 168 and strain 3610, strain 6633 is considered the "wild" parent of strain W23. Strain 6633 was mutated in the laboratory to produce a threonine auxotroph, which is strain 23. In subsequent experiments, strain W23 developed from strain 23 through a reversion to prototrophy in the laboratory (Burkholder and Giles, 1947). W23 does not show any of the hallmarks of truly "wild" strains: it does not swarm or form fruiting bodies when grown on laboratory media. The genome of W23 also differs from the genome sequence of 6633. ATCC 6633 was isolated around 1912, most likely from a soil sample, and deposited in the American Type Culture Collection during the early 1940s. Strain W23 is a derivative of 6633 and is very well domesticated. Although it might be considered domesticated, biofilm formation and other phenomena have not been well studied for strain 6633. Being from different subspecies, the paired strains 6633/W23 and 3610/168 differ significantly in cell wall composition (Zeigler, 2013).

2.4 Bacterial Transport in Porous Media

Many factors are known to affect the transport of biocolloids through porous media, and each factor may impact each organism to a varying degree. Factors affecting colloid transport and fate in saturated porous media include the water matrix (eg. pH, ionic strength, microorganisms, natural organic matter), media characteristics (e.g. mineralogy, grain size, surface roughness, organic content), and (bio)colloid properties (e.g. size, surface charge, surface morphology) (Lytle et al., 2002; Tufenkji et al., 2004).

Transport and attachment of bacterial cells in porous media filtration can be affected by irregular cell shapes (rod or ellipsoid), surface appendages, surface charge and the production of extracullular polymeric substances (EPS). The characteristics of the external shell of a pathogen or colloid can control retention in porous media (Lawrence and Hendry, 1996). EPS production by various bacterial species and strains has not been systematically quantified or qualified, in part due to a lack of adequate standard methods (McLellan, 2012), but EPS plays an integral role in adhesion to solid surfaces. Inter-strain variability has been examined (Bolster et al., 2001; Tufenkji et al., 2007; Castro and Tufenkji, 2008), but warrants further investigation for those biocolloids

that are frequently used as surrogates for pathogen fate and transport. Although differences have been documented between wild-type strains (environmental strains, WT) (Kearns and Losick, 2003) and laboratory organisms (LT), LT strains are more commonly used because they are well studied, easy to work with, and have well documented, successful protocols for growth, purification and use. Controlled studies using LT organisms may produce results that misrepresent natural conditions; inter-strain variability should be considered when using any microorganism. A thorough characterization of the behaviour of LT and environmental bacteria isolates (WT) is essential to clearly understand laboratory-based findings.

Differences between strains of some microbial species can result in significantly different transport and adhesion to porous media. In characterizing mucoid and non-mucoid strains of *Pseudomona aeruginosa,* Martinez-Martinez et al. (1991), found that non-mucoid strains had a higher initial attachment to three surfaces, but different attachment rates and maxima were observed for each of the nine strains examined. Strains were isolated from six different sources and showed varying hydrophobicity. Over time, the mucoid strains were found in greater numbers on the surfaces examined. Once adhered, cell-to-cell communication can lead to the formation of microcolonies and multicellular-type growth which may be facilitated by EPS secrections (Aguilar et al., 2007).

Similarly, in their assessment of two nontoxigenic strains of *Eschericia coli (E. coli*), Castro and Tufenkji (2007), determined that under the experimental conditions investigated, only one strain had potential to be an adequate surrogate for the toxigenic 0157:H7 strain of *E. coli*. They monitored transport through saturated porous media in bench-scale columns and used these data to extrapolate to a field-scale. At low ionic strengths, the non-toxigenic strain theoretically required 5m of travel distance to achieve 99% removal, while the toxigenic strain required an estimated 29m. The results of this study suggested that differences in cell surface charge may account for differential bacterial transport; their calculation of α (collision efficiency) correlated well with zeta potential measurements. Retention of the two colloids was also increased at higher temperatures (22°C compared to 11°C). They concluded that nontoxigenic strains, even from within the same serotype, may not be useful surrogates for pathogenic organisms from the same strain. These results

highlight the inter-strain variability of bacterial characteristics and the need to thoroughly characterize any strains used as surrogates.

Several studies have shown that the presence of EPS increases retention of bacteria in porous media, and treatments to remove EPS result in lower attachment (Bell et al., 2005; Liu et al., 2007; Long et al., 2009; Tong et al., 2010). Bell et al. (2005), examined the retention of wild-type *Psuedomonas putida* modified with cellulose to break down the cellulose macromolecules in EPS and found that attachment was higher for untreated cells. In quartz-packed column transport assays, the calculated collision efficiency dropped for cellulose-treated cells (0.21 ± 0.05) compared to two control groups (~0.40 ± 0.06).

Tong et al. (2010) examined cell transport through 20cm of quartz sand using four representative microorganisms suspended in monovalent NaCl (2.5 to 20mM) and divalent $CaCl_2$ (5mM). They contrasted gram-positive and gram-negative, and motile and non-motile bacteria (*Pseudomonas*: -ve, motile; *Eschericia coli:* -ve, non-motile; *Bacillus subtilis*: +ve, motile; *Rhodococcus*: +ve, non-motile). Breakthrough plateaus were consistently lower for the treated cells under every experimental condition. They determined that untreated bacteria (EPS intact) were removed 5 to 25% more than treated cells (Cation Exchange Resin EPS removal method) regardless of similarities in surface charge. In contrast, Kuznar and Elimelech (2006), cleaved the surface macromolecules from *Cryptosporidium* oocysts with proteinase K (digestive enzyme) and found that deposition was greater for treated oocysts. They hypothesized that electrosteric repulsive forces caused by surface extensions were reduced, leading to higher retention (Kuznar and Elimelech, 2006). These studies highlight the important role EPS plays in bacterial and pathogen deposition and clearly demonstrate the need for inter-strain variability assessment of EPS presence and effects on adhesion.

Many factors can affect bacterial production of EPS including growth conditions, environmental exposure or conditioning, temperature, pH and nutrient availability (Martinez-Martinez et al., 1991; Rose et al., 2007; McLellan, 2012). Martinez-Martinez et al. (1991), were able to culture a non-mucoid producing-variant from a strain of *Pseudomonas aeruginosa* by using solid growth agar. Rose et al. (2007), also found that growth in a liquid medium produced spores that were three times more sensitive to inactivation via heat, in comparison with spores produced using solid growth agar.

Similarly, in their assessment of four bacteria (*Pseudomonas,* two strains of *E. coli,* and *Salmonella*) Soni et al. (2008) found that cells size was largest when the bacteria were grown with a rich growth medium. In an assessment of a marine strain of *Pseudomonas,* Wrangstadh et al. (1990), found that although EPS was produced during cell growth and under starvation conditions, such EPS was differentially bound to the cell. During growth and total nutrient availability, EPS was integral, while during starvation the EPS produced by the cells was loosely bound (peripheral). When peripheral EPS was released by the cells, the viscosity of the medium increased. The mobility of the cells changed over the course of the starvation period; cells lost mobility after 3 hours, but regained it upon release of peripheral EPS after 27 hours of stationary growth. Smets et al. (1999), examined the transport and retention of *Pseudomonas fluorescens* at a range of ionic strengths during exponential, stationary, and decay growth phases (0.02 – 0.2 M carbon-free M9 medium in DI water). The collision efficiency was much higher for cells from the exponential growth phase in comparison with the other two phases. They concluded that ionic strength had no impact on cell attachment but suggested the surface charge of a cell played an important role; the calculated values for α significantly decreased with the centrifugation of exponential-phase cells. They postulated that this was a result of increasing (absolute) zeta potential. Laboratory handling and conditioning of surrogates for use in transport assessments must be thoroughly assessed, as it plays an integral role in the surface properties of the microbial organisms under investigation.

The American Society for Testing and Materials released standardized methods for the propagation of *Bacillus subtilis* in 2011 (ASTM, 2011). However, this procedure is for use with all strains of *Bacillus,* of which there are over 48. As outlined above, the nuances of strain-specific growth requirements, surface characteristics, and EPS production may vary between strains. This variability remains unaddressed; neither the United States Environmental Protection Agency, nor the American Society for Testing and Materials has specified a strain for use during monitoring, performance demonstrations, or riverbank filtration scenarios.

2.4.1 (Bio)Colloid Zeta Potential

Surface charge (measured as electrophoretic mobility) has been highlighted as an important factor describing the stability of a potential surrogate in aqueous environments (Ryan and Elimelech, 1996). Zeta potential (ζ) is a measure of the electrophoretic mobility of a particle, as calculated using the Smoluchowski equation:

$$\zeta = \frac{\eta}{\varepsilon}\mu_e \qquad \text{equation 2.1}$$

where:

ζ= zeta potential
η = viscosity of measuring liquid
ε = dielectric permittivity of measuring liquid

and

μ_e = electrophoretic mobility

The elctrophoretic mobility is defined as the ratio between the velocity of the particle under an applied electric field and the strength of the electric field. Zeta potential is affected by ionic strength, as an increasing concentration of ions in solution causes a compression of the diffuse layer of ions surrounding a particle, partially neutralizing the negative charge (Figure 2.1).

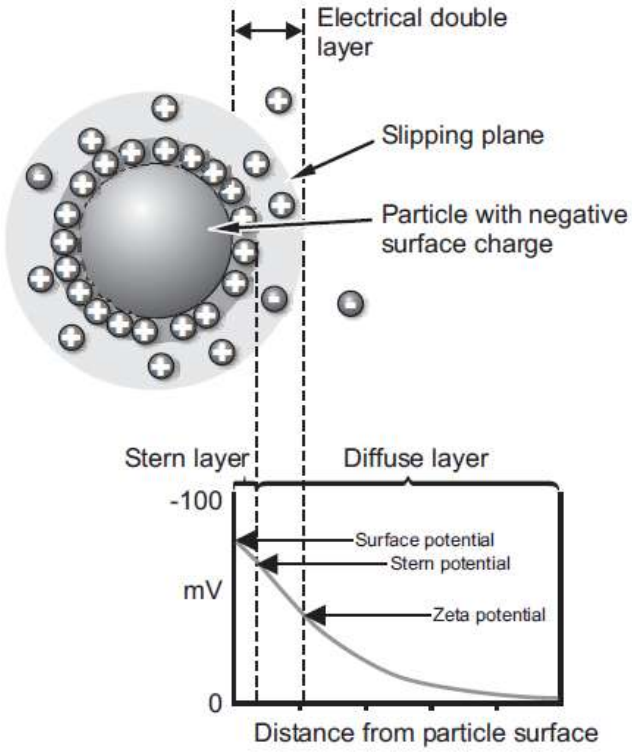

Figure 2.1 Zeta Potential measured at the Outer edge of the Slipping Plane (Malvern, 2004)

The charge at the particle's surface affects the ions distributed in the surrounded region; counterions (ions of opposite charge to that of the particle) will gather close to the particle's surface, creating an electrical double layer. The inner region is known as the Stern layer, and ions here are strongly bound to the particle surface. The outer, more diffuse layer consists of ions that are loosely attached. The boundary between the stable double layer and the unstable ions surrounding it is known as the slipping plane. Ions on the inside of this plane travel and move with the particle, and ions on the outside of this plane do not. The zeta potential is a measurement of the potential that exists specifically at this boundary. The magnitude of the zeta potential is an indication of the stability of the colloidal system.

Most biocolloids, including *Cryptosporidium, E. coli,* microspheres and *Bacillus subtilis* are hydrophobic and have negatively charged surfaces in natural waters (zeta potential between -5 and -50 mV under varying conditions) (Rice et al., 1996; Bustamente et al., 2001; Dai and Hozalski, 2003; Gallardo-Moreno et al., 2003). The zeta potential of *Cryptosporidium* in raw and tap water has been measured in a variety of conditions, with one study measuring an average of -38mV at neutral pH in raw water with a measured conductivity of 400 µS cm^{-1} (Hsu and Huang, 2002) (Table 2.2). The isoelectric point (charge = 0) for oocysts was measured at or near a pH of 3.3 in several studies, which is outside the range of most naturally occurring conditions, as well as most laboratory or field-scale investigations (Hsu and Huang, 2002). The isoelectric point and zeta potential of oocysts can be influenced by source, purification and storage methods used.

Table 2.2 Zeta Potential Measurements (mV) of (Bio)Colloids

(Bio)colloid	Zeta Potential (mV)	Condition Details	Source
Cryptosporidium	-1.5 to -12.5	• pH 6.7 • varied calcium concentration	Dai and Hozalksi, 2003
	0 to -29	• pH range 2 – 12 • NaCl	Bustamente et al., 2001
	-5 to -12	• pH range 4 - 10 • filtered lake water	Rice et al., 1996
	a) -35 b) -10	a) Distilled water, neutral pH b) pH 3.5	Ongerth et al., 1996
	a) -17 to -30 b) -3 to -8 c) -6 to 0	a) pH 5.5-5.7 in 1mM KCl b) 100mM KCl c) 0.1 to 100mM $CaCl_2$	Kuznar and Elimelech, 2004
	-38	• Neutral pH	Hsu and Huang, 2002
Bacillus subtilis	-8	• Strain CB2, conditions unspecified	Gannon et al., 1991
	-15 to -20	• pH 5 to pH 11 in filtered lake water	Rice et al., 1996
	-36	• strain CU 4519, unspecified	Gannon et al., 1991
	-15	• strain CB3	Gannon et al., 1991
	a) -18 b) -58	• 0.01M NaCl a) pH = 2 b) pH = 9	Sharma et al., 1985
Carboxylated MS	-7.4 to -50.2	• 5.0µm, pH 6.7, varied calcium concentration (10^{-6} to 10^{-1} M)	Gannon et al., 1991
Carboxylated MS	-83.5	• 1.0µm Yellow-Green	Bradford et al., 2004
	-85.5	• 3.2µm Yellow-Green • pH and ionic strength not specified	Bradford et al., 2004
	a) -104 b) -104 c) -104 d) -78 e) -64	a) IS = 6 mM NaCl b) IS = 31mM NaCl c) IS = 56 mM NaCl d) IS = 81 mM NaCl e) IS = 106 mM NaCl • 1.1 µm Yellow-Green	Bradford et al., 2007
	-76	• IS = 6 mM NaCl • 3.0µm Yellow-Green	Bradford et al., 2007
	-32.33	• 1.0µm, pH 6.33	Sharma et al., 1985

Zeta potential is affected by pH, and the contents and concentration of the suspending solution. Lytle et al. (2002) investigated the zeta potential of oocysts and found that while suspended in phosphate buffered saline (PBS) solution, the zeta potential became more negative as pH increased from 2 to 8. The changing pH had more of an effect on oocysts suspended in low concentrations of PBS (0.915mM versus 91.5mM) (Lytle et al., 2002). During their study, Dai and Hozalski (2003), found that oocysts coated in natural organic matter (NOM) had a zeta potential near -30mV in 10^{-5}M Ca^{2+}, but this became less negative as calcium concentration increased (pH was held constant at 6.7). Uncoated oocysts had a zeta potential of -10mV and were less affected by the changing concentration of calcium ions. In natural waters, the absolute value of zeta potential should generally decrease with increasing ionic strength, and increase as pH becomes more basic (Rice et al., 1996; Ahimou et al., 2001). The theory of electrical double-layer compression suggests a decrease in zeta potential with increasing ion concentrations in solution. A less negative charge may result in higher attachment rates (Kim et al., 2008b). However, other factors can also influence negative charge: for example, Kunzar and Elimelech, (2004), found that protein hairs extending from the surface of *Cryptosporidium* oocysts were acting as a repulsive barrier, increasing the negative charge and decreasing deposition.

Although surface charge may play an important role in deposition kinetics, Gannon et al. (1991), studied the transport of 19 strains of Bacteria, including *Bacillus,* through Kendaia loam and found no correlation between deposition and hydrophobicity or surface charge. They tested ten strains for the presence of flagella and found no correlation between flagellated bacteria and transport (Gannon et al., 1991). They concluded that cell size was the most influential factor affecting transport, but postulated that several characteristics of the cell must contribute to determining overall transport through saturated porous media. Cells less than 1 µm travelled furthest in their study.

Laboratory handling has been shown to affect the zeta potential of bacterial cells. Soni et al. (2008), also found that nutritionally-deprived *E. coli* cells had a more negative zeta potential compared to those grown in a nutrient-rich medium, when each was suspended in filtered municipal drinking water (pH of 8.4). Smets et al. (1999), found that centrifugation increased the negative charge associated with a cell (*Pseudomonas*

fluorescens), resulting in only ~10% attachment in a packed glass bead column in comparison with 40% attachment for cells that did not undergo centrifugation. Centrifugation reduced α by a factor of 3. *Bacillus subtilis* purification methods call for repeated centrifugation, which may have cumulative effects on the spore surface charge.

Laboratory treatments may also affect the outer layer of a spore. Sharma et al. (1985), treated *Bacillus subtilis* spores with a variety of chemicals (sodium pyrophosphate, heparin, ferrochrome lignosufonate, polyacrilic acid) and found that zeta potential varied with chemical treatment. Untreated cells measured between -18 and -58mV at pH 2, 3 and 9 and zeta potential values and were always less negative when compared to treated cells, except in one case after treatment with sodium pyrophosphate at a pH of 6. More negative bacterial cell surface charge resulted in less adhesion during column transport studies (flow = 10mL/hour, 0.01M NaCl). Using two enterococci strains at cell concentrations of 10^7 to 10^8 cells/mL, Gallardo-Moreno et al. (2003), also saw a decrease in zeta potential with increasing ionic strength of the suspending medium. They observed a doubling of zeta potential at 37°C when the strain was suspended in PBS (-16mV) compared to suspension in KPi (-32mV). Generally, increasing the temperature (22°C to 37°C) caused all samples to become less negatively charged. Collectively, the literature indicates that bacterial cells present in drinking water can exhibit significant differences in zeta potential based on the physiological state as determined by laboratory methodology and are greatly affected by the environment in which they are measured (Smets et al., 1999; Gallardo-Moreno et al., 2003; Soni et al., 2008).

2.5 Colloid Filtration Theory

Bacillus subtilis spores are colloidal in size (several nanometers up to 10 µm) as are the oocysts of *Cryptosporidium*. The physicochemical properties of the spore such as size, shape, cell surface characteristics, and surface charge may impact transport through granular media (collectors) as will environmental conditions such as ionic strength, pH, and temperature. The mechanisms of particle removal during passage through saturated, spherical, porous media are well described by classic colloid filtration theory. Colloid removal by porous media occurs in two main steps: transport to a collector (media grain) surface, and reversible or irreversible physicochemical

attachment to the surface (Yao et al., 1971, Elimelech, 1992; Tufenkji and Elimelech, 2004). A particle traveling in the fluid matrix is subject to diffusion, sedimentation, and interception (Figure 2.2) (Yao et al., 1971), which are physical processes governing whether a colloid with collide with a collector (contact).

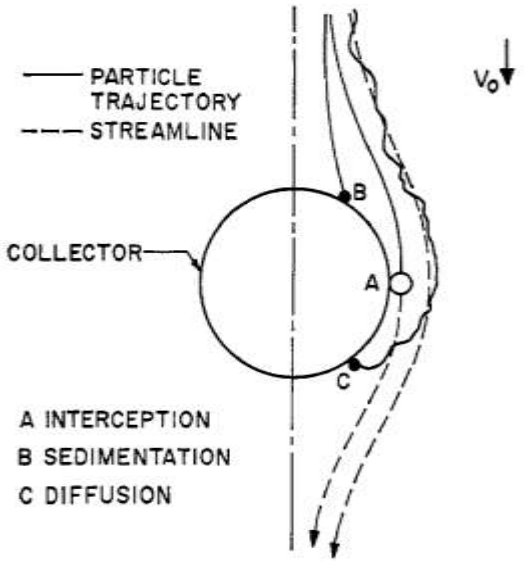

Figure 2.2 Transport Mechanisms in Water Filtration (Yao et al., 1971)

Colloids can be intercepted by collectors, travel to collectors via Brownian motion (random diffusion due to thermal activity), or settle onto collector surfaces by sedimentation. Diffusion describes a particle's random movement due to Brownian motion while in suspension and is inversely related to particle size (equation 2-2).

$$D_p = \frac{kT}{3\pi\mu d_p} \qquad \text{equation 2-2}$$

Where: D_p = particle diffusivity in water
K = Boltzman constant (J/K)
T = absolute temperature (K)
μ = viscosity of water (g/cm/s)
d_p = particle diameter (cm)

Smaller pathogens and surrogates (<3μm) will be affected to a greater extent by diffusion to a collector surface, relative to larger colloids. Interception is based upon a

particle's tendency to stay in motion, calculated using the ratio of particle diameter to collector diameter (equation 2-3).

$$\eta_0 \propto \frac{d_p}{d_c} \qquad \text{equation 2-3}$$

This equation assumes spherical collectors, which may not hold for all alluvial sands and gravels (Tufenkji 2007). As the size of a particle (colloid) increases relative to the size of a collector, the likelihood that the colloid will deviate from its path due to advective flux near the grain surface also increases. The surfaces of larger colloids are therefore more likely to come into closer contact with a collector surface before the surface of a smaller colloid. Sedimentation is also proportional to colloid size and is the downward motion of a particle due to differences in density (equation 2-4, Stoke's law).

$$v_s = \frac{(\rho_p - \rho) g d_p^2}{18\mu} \qquad \text{equation 2-4}$$

Where:

V_s = settling velocity (cm/s)
g = gravity (cm/s^2)
ρ = density (g/cm^3)

Settling velocity is proportional to the square of the particle density, which is assumed to be greater than the density of water. Stoke's law assumes the particles are spherical and rigid which may not be valid for (bio)colloids which often have rough, malleable surfaces, surface appendages and can be spherical, rod or ellipsoid-shaped. During an assessment of bacterial transport, Weiss et al. (1995) used 14 environmental isolates in columns packed with clean quartz sand to determine the impact of bacterial shape on filtration. They determined that larger, rod-shaped bacterial were preferentially removed from solution (cell width to cell length ratio) during passage through short quartz-packed columns. They also quantified the size and shape of the bacteria in the influent and effluent, finding a higher proportion of small spherical bacteria in the effluent, indicating preferential removal of rod shaped bacteria, or an increased post-transport frequency of coccoid-shaped cells. Gannon et al. (2001) examined the transport of 10 strains of bacteria, including *Bacillus*, through 5cm of packed sand at 3°C and found that bacteria smaller than 1µm showed the most transport, regardless of bacterial strain. They determined that no relationship was evident between the

genera of the strain and their mobility in 5cm of loam. The experimental conditions involved dry packing, petrolatum coated columns and reverse filling the chamber with water. In their assessment of *Clostridium* spore transport, Schijven et al. (2003), found that spores were removed to a greater extent than smaller-sized virus surrogates (MS2) in spite of theory suggesting otherwise. However, they also suggested that due to lack of inactivation, long-lived spores were reversibly attached and should hypothetically breakthrough to the effluent eventually.

Particle contact with collector surfaces is mathematically described by collision efficiency, or η_0, which is impacted by the three processes described above. Colloids smaller than 3µm will be affected to a greater extent by diffusion, whereas the processes of sedimentation and interception have a greater impact on larger colloids. Additivity was demonstrated by Yao et al. (1971), and used to describe a single collector efficiency (Figure 2.3). The single-collector efficiency model empirically predicts a colloid falling in the size range of approximately 1-3µm will be minimally affected by sedimentation and interception, as well as diffusion, and demonstrate the lowest contact efficiency, traveling the furthest in porous media (Tufenkji and Elimelech, 2004; Kim et al., 2008a). The conceptual model includes a clean-bed assumption in which no deposition between pore spaces has altered fluid flow, as well as steady state colloid concentration system that is not subject to hydrodynamic forces (Lawrence and Hendry, 1996). These theoretical assumptions are invalid for experimental column systems into which colloids are being introduced and RBF systems which are not limited to perfectly spherical collectors and where pore spaces are in constant flux due to biological activity (Tufenkji 2007). The diameter of collectors in an RBF system may also be continuously changing as blocking occurs and biofilms develop.

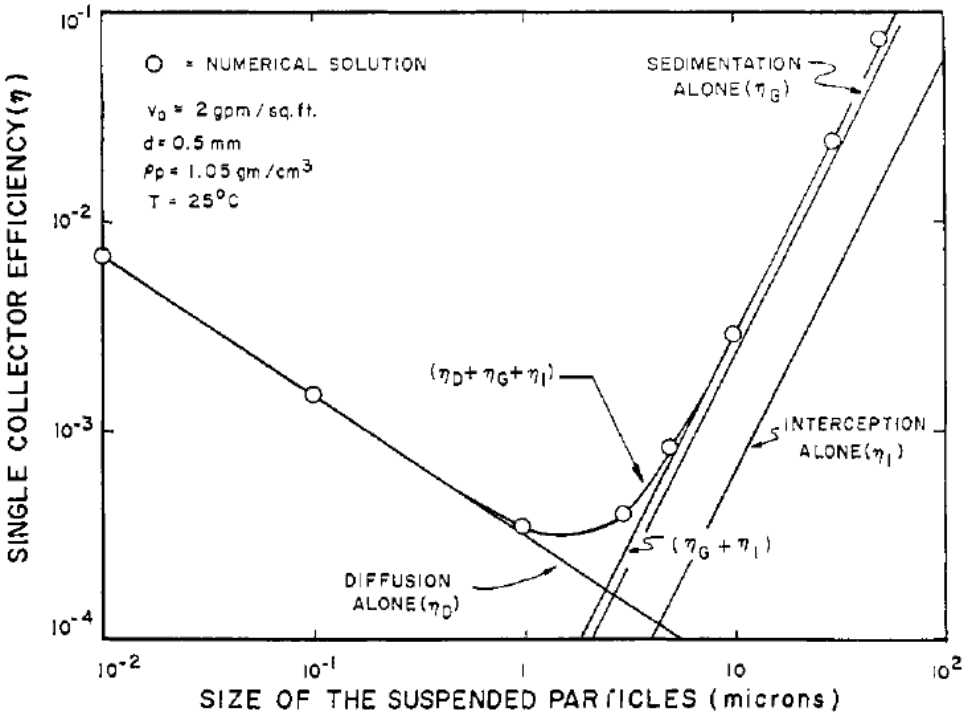

Figure 2.3 Conceptual Model of Single Collector Efficiency (Yao et al., 1971)

Once particles (colloids) in the fluid matrix reach the surface of a filter or media grain (collector) the second step in classical filtration theory (CFT) involves colloid removal (attachment, attenuation, retention) via physicochemical attachment (McDowell-Boyer et al., 1986; Tufenkji and Elimelech, 2005). Probability of attachment is then the product of the single-collector contact efficiency (colloid contact efficiency, collector efficiency, transport contact efficiency) and the deposition, attachment, collision, or sticking efficiency (α) (Equation 2-5) (Elimelech, 1994).

$$\eta = \alpha\eta_0 \qquad \text{equation 2-5}$$

Attachment efficiency (α) is dependent on the interaction energy between the colloid and the collector and is usually determined using DLVO theory (Derjaguin-Landau-Verwey-Overbeek), which combines Lifshitz-Van der Waals interactions with electrostatic double layer interactions to determine if deposition is favourable or unfavourable (Derjaguin and Landau, 1941; Verwey and Overbeek, 1948). For column experiments, the α parameter can be back-calculated if a theoretical value for η_0 has been determined (equation 2-6) but involves a clean-bed assumption and excludes

factors that have been shown to have a significant impact on biocolloid transport including ionic strength and flow rate (Kim et al., 2008; Schinner et al., 2010).

$$\ln \frac{C}{C_0} = -\frac{3}{2}(1-\varepsilon)\alpha \eta_0 \left(\frac{L}{d_c}\right) \quad \text{equation 2-6}$$

where:

C = pseudo steady-state effluent Concentration
C_0 = Influent concentration
ε = porosity
L = column length
D_c = median grain diameter

Sticking efficiency, α, may increase with increasing ionic strength, but comparisons of α values calculated under different experimental conditions have limited use. The α parameter is based on a conceptual model and is not a predictive tool. Bell et al. (2005), calculated α for the retention of *Psuedomonas putida* in columns filled with either quartz or glass beads and found higher retention in the glass beads (α ~0.4 for quartz). In RBF situations, quartz grains are common bed material, and media heterogeneity is much higher than in a controlled laboratory environment or plant filters.

Recent studies have demonstrated deviations from standard DLVO theory (Sharma et al., 1985; Tufenkji and Elimelech, 2005; Schinner et al., 2010; Tong et al., 2010). The electrostatic double layer describes the ions surrounding a colloid in solution: a tightly bound inner layer fixed to the surface of a colloid, and a loosely attached, diffuse layer of ions that move about more freely in the liquid matrix. The 'plane of shear' denotes the separation between these two layers, and is used to determine the strength of the diffuse layer which is measured as electrophoretic mobility (and used to calculate zeta potential which will be discussed in further detail in Section 2.4.2). The electrostatic double layer is significantly affected by ionic strength, as a larger number of counterions in solution act to compress the diffuse layer. Collision efficiency is used to qualitatively gauge the effects of solution chemistry, collector characteristics and particle chemistry on overall colloid removal. Collector efficiency describes the ratio of overall particle deposition rate onto a collector to the particles travelling toward that area of a collector (Ryan and Elimelech, 1996). Sticking efficiency describes the

ratio of particle adhesion to a collector, relative to the total number of collisions between colloids and a given collector

Under CFT, filtration is often characterized as "favourable" or "unfavourable" to describe the attachment conditions for (bio)colloids traveling through the saturated medium. Unfavourable conditions arise when electrostatic double-layer negative forces dominate, resulting in a net repulsive force that overpowers attractive Van Der Waals forces. Repulsive forces, caused by like charges or steric repulsion, are a function of the separation distance between a colloid and a particle's surface, and result in an energy barrier that must be overcome to attach at the second (weak) energy minimum (5-10nm) or the primary energy minimum (strong attachment, ≤1nm) (Figure 2.4).

Favourable conditions describe a situation in which repulsion is minimal, or attractive double-layer forces govern separation distances and there is no energy barrier to attachment (Dai and Hozalski, 2003; Tufenkji and Elimelech, 2004). Favourable conditions can be simulated by high ionic strength solutions or acidic conditions, which act to compress the electrostatic double layer at the surface of most biocolloids. RBF scenarios with high ionic-strength groundwaters, or where riverbed mineralogy leads to the dissociation of positive ions such as iron or manganese, can encourage deposition (Bolster et al., 2001). Schijven and Hassanizadeh (2000), found that viruses were able to attach to the positively charged minor surface fraction of a soil grain, even when general conditions for attachment were unfavourable. At the plant level, net repulsive forces can be overcome through the addition of coagulants and flocculants (positively charged) or changing solution chemistry to enhance attachment, options which are unavailable during natural RBF.

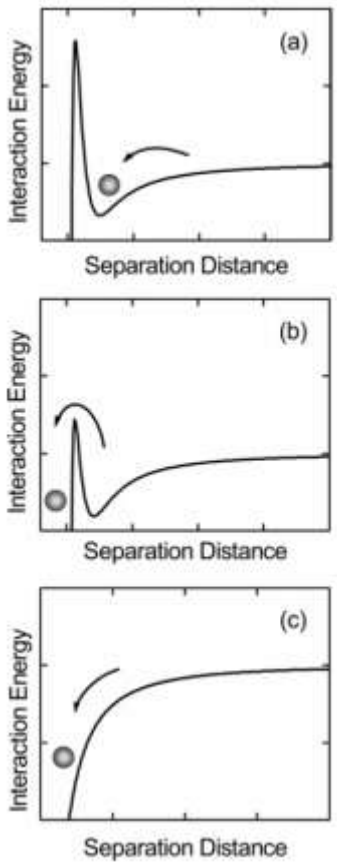

Figure 2.4 Schematic of Interaction Energy Profiles (Tufenkji and Elimelech, 2004) a) particles retained at the second energy minimum b) particles overcoming repulsion to deposit at the primary energy minimum, and c) particles depositing in favourable conditions

Colloid removal by size exclusion (typically referred to as straining) also contributes to concentration reductions during passage through porous media. For spherical collectors, a colloid/collector diameter ratio greater than 0.154 should exclude colloids from passage into porous media, resulting in the accumulation of a 'cake' layer on top of the filter media (Herzig et al., 1970). Straining occurs at a lower colloid/collector ratio, which allows colloids to pass into the media but they are strained, or retained in the small pore spaces where collector grains are just adjacent to one-another (Bradford et al., 2004; Bradford et al., 2007). It is simply the physical entrapment of particles in down-gradient pore throats through which they are too large to pass (McDowell-Boyer, 1986). Such locations are optimal for weak attraction because hydrodynamic forces are reduced, but may also lead to reversible attachment/ detachment. Straining contributes to significant colloid removal even if CFT suggests no removal should occur (Tufenkji et al., 2004). Straining may become a significant factor in colloid retention at colloid/collector diameter ratios greater than 0.005 (Bradford et al., 2005)

although grain angularity and ionic strength may also contribute (Tufenkji et al., 2002; 2004).

Current colloid attachment models are unable to account for the extremely variable retention of biocolloids in porous media, part of which is due to the heterogeneity of living microorganisms and their vulnerability and adaptability to solution chemistry (pH, ionic strength, salinity, dissolved oxygen) (Fontes et al., 1991; Lawrence and Hendry, 1996). Microbes can also be affected by natural parameters including temperature, sunlight, nutrients and turbidity (Ahmad et al., 2009). Although the use of surrogates avoids some of the complexities of working with pathogenic microorganisms, biocolloids, as living organisms, will also respond to each experimental environment. The surface properties of microorganisms can vary based on source, age, assay method used, and growth media or preparatory solutions used, which will further contribute to variability in data (Gannon et al., 1991; Lawrence and Hendry, 1996; Chauret et al., 2001; Dai and Hozalski, 2003; Rose et al., 2007; Tufenkji 2007; Bolster et al., 2009). Many factors can affect filter performance and (bio)colloid removal, including, but not limited to:

Biocolloid properties:

- size, shape and rigidity
- surface roughness or presence of appendages
- surface chemistry
- density
- population heterogeneity
- species and metabolic rate
- chemotaxis or mobility/motility
- cell attachment/biofilm growth/ colony and fruiting body formation

Water Quality:

- pH
- ionic strength
- temperature
- presence of natural organic matter or suspended solids
- seasonal variability

Filter Media characteristics:

- grain size
- filter depth
- uniformity coefficient
- surface chemistry and surface heterogeneity
- porosity
- grain shape
- pore geometry

Operational Parameters:

- loading rate
- influent water quality
- infiltration of surface waters

Theoretical modeling of colloid deposition onto porous media makes simplifying assumptions and avoids the inherent complexity of microbial behaviour under non-steady-state conditions, and excludes pore structure heterogeneity, straining, electrostatic interactions, and other important factors (Yao et al., 1971; Sharma et al., 1985; Tufenkji et al., 2004; Bradford et al., 2007). Currently, unfavourable attachment conditions, more frequently encountered in nature, are also more likely to deviate from classic colloid filtration theory (Tufenkji 2007).

2.5.1 Media Characteristics

The mineralogy, size, shape and texture of porous media can affect colloid deposition to media surfaces during filtration. In well-controlled laboratory experiments, round collectors are often used (Harvey et al., 1995; Jewett et al., 1995; Hsu et al., 2001; Kim et al., 2008b) in contrast to the heterogeneous sand grains found in natural subsurface environments (Bolster et al., 2001; Côté, 2004; Knappet et al., 2008). Hsu et al. (2001), compared glass and polystyrene beads and found *Cryptosporidium* removal comparable between the two collectors. They concluded that the ionic strength of the solution had more of an impact than either collector material. Tchio et al. (2003) compared sand with anthracite and found no significant difference in removal over a wide variety of parameters at pilot-scale. Tong et al. (2006), studied the deposition behaviour of five sizes of carboxylate-modified microspheres using porous media columns packed with either spherical soda-lime glass beads or quartz sand. Under favourable conditions MS deposition on quartz was a factor of 2 higher than on glass beads; under unfavourable conditions the difference in deposition was an order of magnitude (510µm collectors). They concluded that pore geometry was a critical factor in colloid retention, providing greater surface area for attachment. Aquifer sands can be highly irregular in shape, ranging from angular to well-rounded as they are weathered and abraded by the elements and softer minerals dissolve. This creates a variety of pore geometries and sizes between the media grains as they fit together; this may be more conducive to deposition for the various bacterial shapes (Tufenkji et al., 2002, 2004; Kim et al., 2008a; Haznedaroglu et al., 2009). Tufenkji et al. (2004),

also suggested that grain shape irregularity contributed "considerably to the straining potential of the porous medium" but a comparison of (bio)colloid removal using well-rounded quartz grains and very angular quartz grains of the same size would confirm these suggested effects.

2.5.2 Grain Size

As media grain size decreases there will be an increase in colloid removal (Bradford et al., 2004; Tufenkji et al., 2004; Kim et al., 2008b). Smaller diameter media result in smaller pores, more surface area upon which colloids can attach and more physical straining of larger (bio)colloids (Lang et al., 1993; Hunt et al., 2003; Tufenkji et al., 2004; Knappett et al., 2008). Kim et al. (2008a), found that *Bacillus cereus* spore transport increased by 82% when grain size doubled. However, in a study conducted by Bradford et al. (2004), comparing a variety of median grain sizes, no substantial difference was observed in the breakthrough of latex particles for grain sizes of 0.36, 0.24 and 0.15mm. In 0.5mm diameter sand media, straining should be significant for colloids larger than 2.5µm, which should affect retention of *Cryptosporidium* but not bacterial-sized microspheres or *Bacillus* spores. At low ionic strengths (<30mM), the impact of grain size on particle removal may be less significant due to increased repulsion (Bradford et al., 2007). Grain size selection can directly impact colloid removal.

Lawler and Nason (2006), concluded that loading rate must be considered in conjunction with effective size (D_{10} = ES) as it can alter the relationship between media size and filter performance. This was in agreement with findings from Bradford et al. (2007), who determined that doubling the velocity (0.2cm/min to 0.45cm/min) caused a 35% increase in effluent particle concentrations regardless of grain size. Straining may play a more important role in (bio)colloid retention at lower flow rates. Tchio et al. (2003), concluded that an ES of 0.4mm offered the best and most economical option for kaolin clay particulate removal by direct filtration at a plant scale. Media characteristics typical in riverbeds and shallow aquifer systems include a D_{50} ranging from 0.2 to 1.25mm (Harvey et al., 1995; Pang et al., 1998; Côté, 2004) and many laboratory-scale filtration studies use media within this range (Weiss et al., 1995; Ongerth and Pecoraro, 1995; Dai and Hozalski, 2003; Bradford et al., 2007; Tufenkji, 2007; Kim et al., 2008b).

2.5.3 Uniformity Coefficient

The heterogeneity of media size is described by the uniformity coefficient (UC) which is equal to D_{60}/D_{10}. A lower UC means a more uniform grain size distribution, or a homogeneous sample. Filters with high UC will have lower porosity, as the smaller pore spaces between large grains are filled by media with a smaller grain size. In their study of filter performance under different conditions, Tchio et al. (2003), found that changes in UC (1.3 to 1.5) had only minor effects on particulate removal at 20°C, while a large change in effective size (D_{10}) had a larger impact on pilot plant filter performance (0.4mm to 2.0mm). Small variations in the UC parameter are not expected to significantly affect biocolloid removal. A typical UC in riverbed materials of alluvial sands or shallow aquifer materials would be near or approximately 1.9 (Harvey et al., 1995; Pang et al., 1998; Côté, 2004)

2.5.4 Ionic Strength

Ionic strength and pH can change colloid surface chemistry and the interactions between collectors and colloids, reducing energy barriers and either increasing or decreasing attachment (Tufenkji et al., 2006; Tufenkji and Elimelech, 2005). Several studies have demonstrated higher colloid retention at higher ionic strengths (Scholl et al., 1990; Fontes et al., 1991; Gannon et al, 1991b; Mills et al., 1994; Schijven and Hassanizadeh, 2000; Compere et al., 2001; Hsu et al., 2001; Kuznar and Elimelech, 2004; Bradford et al., 2007; Torkzaban et al., 2008). As the concentration of dissociated charged ions increases (higher ionic strength), ions in solution will arrange themselves near collector surfaces, neutralizing electrostatic repulsive forces and allowing Van Der Waals attractive forces to dominate. Hsu et al. (2001), found a 30% increase in removal of *Cryptosporidium* as ionic strength increased from 0.01M to 1M $NaClO_4$. Jewett et al. (1994), made the same observation: collision efficiency (α) of *Pseudomona fluorescens* decreased 90% when ionic strength was reduced from 10^{-1} to 10^{-5} M NaCl in a column packed with silica beads. In contrast, pH changes from 5.5 to 7.0 caused no significant change in calculated α. Bolster et al. (2001), also observed a decrease in bacterial removal in clean silica sand with a reduction in ionic strength from 10^{-1} to 10^{-2} M KCl in a 5cm column. The impact of ionic strength can be partially mitigated by sands with positively charged surfaces, such as high metal-oxide content, which offers a positively charged attachment site for (bio)colloids (Bolster et al., 2001). Low ionic strength conditions create unfavourable attachment conditions; Torkzaban

et al. (2008), found that attachment was negligible at or below an ionic strength of 50mM KCl. Controlled laboratory column experiments can demonstrate (bio)colloid removal in a range of conditions during filtration through granular media using both favourable and unfavourable deposition conditions (Mills et al., 1994; Jewett et al., 1994; Schijven and Hassanizadeh 2000; Bolster et al., 2001; Tufenkji et al., 2004).

Higher ionic strength solutions can also affect the EPS of a bacterial cell, impacting attachment in turn. Using transmission electron microscopy (TEM) imaging, Frank and Belfort (2003), and Kuznar and Elimelech (2004), found that at low ionic strengths the EPS of *Pseudomona atlantica* extended further into the bulk fluid matrix, while at higher ionic strengths the EPS polymers coiled and stayed close to the cell wall. Camesano and Abu-Lail (2002), also concluded that surface polymer chains of *Psuedomonas putida* remained extended in water when compared with salt solutions; they suggested the heterogeneity of biopolymer properties for one bacterium, and within a bacterial population was vast and should be considered when modeling adhesion. These results indicate that changes in ionic strength can impact the EPS of a (bio)colloid. Changes in cell polymers can also impact cell shape and diameter. *Bacillus subtilis* is a flagellated, motile bacterium that produces EPS, so it is likely that higher ionic strengths will have similar effects (Gannon et al., 1991).

Low ionic strength solutions (0.1mM KCl) may represent groundwater conditions following a rainfall event that has diluted ion concentrations. Tufenkji and Elimelech (2004), noticed no discernible difference in breakthrough curves of model latex particles (3.0µm), at 10mM and 3mM KCl, while above 10mM KCl, ionic strength had a large impact on retention of the particles. Ionic strengths ranging from 0.10 to 10mM may represent riverbank filtrates (Conductivity ~600µS/cm, Côté, 2004), while ranges up to 1M replicate surface waters or GWUDIs. Monovalent potassium chloride (KCl) is often used as an artificial groundwater during pathogen and surrogate bench scale investigations, with concentrations ranging from 0.1mM to 5M (Bolster et al., 2001; Frank and Belfort, 2003; Kuznar and Elimelech, 2004; Castro and Tufenkji, 2007; Knappett et al., 2008).

2.6 Transport Experiments

Colloid filtration theory describes removal of colloids as log-linear with distance; column scale and field scale investigations monitor removal of (bio)colloids over set

distances. Bench-scale laboratory columns replicate naturally occurring condition, matching aquifer materials, flow rates, and groundwater characteristics. The naturally occurring residence time of surface water passing through the subsurface during RBF is determined by local conditions and usually varies from 5 to 100 days (Kuehn and Mueller, 2000). Subsurface stratigraphy, hydraulic gradient and flow pathways, as well as well pumping rates will affect groundwater movement during RBF; it has been documented in the range of 0.1 to 1m per hour (Table 2.3), with faster flow in areas of poorly sorted sediments or unconfined aquifers (Starr and Cherry, 1994; Harvey et al., 1995). Schijven et al. (2003), determined a transport velocity of bacteriophages and *Clostridium* spores reaching 1.5m/day in a sand dune aquifer. In contrast, a study conducted by Pang et al. (2005), monitored the transport of *Bacillus subtilis* spores and determined a colloid velocity of 63m/day, or 2.625m/hour. Flow rates were reported to have the greatest effect on the movement of *Bacillus cereus* spores (as compared with grain size and pH) by Kim et al. (2008a). They concluded that the increase in water velocity increases the hydrodynamic forces acting upon the soil surfaces, thereby reducing interparticle bonding, decreasing adhesion and increasing particle mobility. Many column-scale examinations vary flow rate to provide a range of potential results that might be expected in the field; Kim et al. (2008), used flow rates of 1.3mL/min and 3.0mL/min, finding spore movement increased by 71% at the higher rate. Appropriate loading rates, encountered at field-scale, should be used during laboratory-scale assessments of (bio)colloid transport.

Table 2.3 Flow Rates/(Bio)Colloid travel times for several riverbank filtration sites in Europe

Location	Rhine River	Meuse River	Meuse River		
Travel distance (m)	30	25	13	25	150
Travel Time (days)	15	63	7	18	43
Loading Rate (m/hour)	0.083	0.017	0.1	0.1	0.1

Adapted from Tufenkji et al., 2002

Full-scale field investigations are time consuming and expensive; controlled laboratory column investigations can elucidate (bio)colloid attachment behavior during transport

through saturated porous media. A variety of collectors can be assessed (Harvey, 1995; Hsu et al., 2001; Knappett et al., 2008), at a variety of ionic strengths (Scholl et al., 1990; Fontes et al., 1991; Gannon et al, 1991b; Mills et al., 1994; Schijven and Hassanizadeh, 2000; Compere et al., 2001; Hsu et al., 2001; Kuznar and Elimelech, 2004; Bradford et al., 2007; Torkzaban et al., 2008) under different flow regimes (Gannon et al., 1991; Kim et al., 2008). Most knowledge of bacterial transport through geologic material must be gained through tightly controlled, reproducible bench-scale column assessments (Lawrence and Hendry, 1996). An understanding of the factors influencing pathogen and surrogate removal during filtration through saturated porous media can allow utilities to exploit or enhance specific mechanisms to improve and maximize the performance of natural and engineered filters.

2.6.1 Research Needs

An examination of the literature related to the use of bacteria, and specifically *Bacillus subtilis,* as surrogates during field and laboratory-scale filtration investigations, reveals a need to compare the transport and retention of spores of various *Bacillus subtilis* strains during filtration through saturated porous media. A thorough assessment of the suitability of *Bacillus subtilis* spores as conservative transport surrogates for *Cryptosporidium* oocysts would also be beneficial. This research aims to determine if the inter-strain variability of *Bacillus subtilis* will result in quantifiable differences in spore transport at the laboratory scale under conditions representative of riverbank filtration.

Chapter 3 Methods

3.1 Research Approach

To address the research needs identified above, the transport behaviour of the aerobic endospores of *Bacillus subtilis* was studied at bench-scale by suspending spores in an artificial groundwater (AGW) solution and passing the suspension through saturated granular media in a down-flow configuration. The goal of the work was to determine the impact of strain selection on spore and pathogen surrogate removal at loading rates and ionic strengths representative of riverbank filtration (RBF). Granular media filtration of four strains of *Bacillus subtilis* spores was quantified at two levels of ionic strength (Table 3.1). The experimental design is outlined (Section 3.1.2) and the detailed methods for the preparation of the artificial groundwater, the spore suspensions, and the granular media are presented in Sections 3.3, 3.4, and 3.5 respectively. Column materials and methods are presented in section 3.2, and data analysis approaches are described in section 3.6.

Table 3.1 Experimental Design

Experiment #	Colloid	Colloid Code	Trials	Conditions
1	Nile Red Microspheres 1.1 µm diameter	NR MS 1.1	1	0.1mM KCl
2	Yellow-Green Microspheres 4.5 µm diameter	YG MS 4.5	1	0.1mM KCl
3	*Bacillus subtilis* subspecies *subtilis* wild-type strain 3610	3610	1 2 3 4	0.1mM KCl 0.1mM KCl 10mM KCl 10mM KCl
4	*Bacillus subtilis* subspecies *spizizenii* laboratory-type strain W23	W23	1 2 3 4 5	0.1mM KCl 10mM KCl 10mM KCl 0.1mM KCl 0.1mM KCl
5	*Bacillus subtilis* subspecies *subtilis* laboratory-type strain 168	168	1 2 3 4 5	10 mM KCl 10 mM KCl 0.1mM KCl 10 mM KCl 0.1mM KCl
6	*Bacillus subtilis* subspecies *spizizenii* wild-type strain 6633	6633	1 2 3 4 5 6	10 mM KCl 0.1mM KCl 0.1mM KCl 10 mM KCl 10 mM KCl 10 mM KCl

3.1.2 Experimental Design

Experiments were designed to demonstrate whether laboratory-type (LT) and wild-type (WT) strains of *B. subtilis* spores were similarly removed by saturated porous media within a controlled environment. Spores of each *B. subtilis* strain were suspended in an AGW solution and passed through duplicate filtration columns (Figure 3.1); each experimental condition was studied in duplicate or triplicate. Spore concentrations of 1.0×10^6 CFU/mL were targeted, but varied with each trial (Table 3.3). Spore retention at unfavourable and favourable deposition conditions was evaluated by using two ionic strengths. The transport of *B. subtilis* spores in sand was compared to that of two sizes of polystyrene microspheres. The experimental conditions utilized during this investigation are summarized in Tables 3.1 and 3.2 and illustrated in Figures 3.1 and 3.2.

Figure 3.1 Bench-Scale Column Apparatus Set Up (not in operation)

Table 3.2 Summary of Experimental Conditions Utilized during Spore Transport Studies

Experiment #	Strain/ MS Size	# of Columns	Ionic Strength (KCl)	Feed Suspension Volume (mL)	Calculated Influent Concentration (CFU/mL)
1	1.1 µm	1	0.1mM	2000	$9.27x10^5$
2	3610	2	0.1mM	2000	$1.06x10^6$
3	W23	2	0.1mM	2000	$4.10x10^5$
4	W23	2	10mM	2000	$4.30x10^5$
5	W23	1	10mM	2000	$1.09x10^6$
6	W23	1	0.1mM	2000	$4.26x10^5$
7	W23	2	0.1mM	2000	$3.53x10^5$
8	3610	2	0.1mM	2000	$1.60x10^5$
9	3610	2	10mM	2000	$1.67x10^5$
10	3610	2	10mM	2000	$2.00x10^5$
11	168	2	10mM	2000	$1.40x10^3$
12	168	2	10mM	1500	$1.05x10^6$
13	168	2	0.1mM	1500	$8.27x10^5$
14	168	2	10mM	1500	$6.51x10^5$
15	6633	2	10mM	1500	$1.01x10^6$
16	4.5 µm	2	0.1mM	1500	$1.0x10^5$
17	6633	2	0.1mM	1500	$5.80x10^5$
18	6633	2	0.1mM	1500	$2.75x10^6$
19	6633	2	10mM	1500	$1.65x10^6$
20	6633	2	10mM	1500	$1.04x10^6$
21	168	2	0.1mM	1500	$1.75x10^5$
22	6633	2	10mM	1500	$9.31x10^5$

Table 3.3 *Bacillus subtilis* Spore Feed Suspension Concentrations

					Target concentration = 1.00×10^6 CFU/mL
Bacillus subtilis Strain	N	Mean	SD	Maximum	Minimum
3610	4	354287	372595	912857	151481
W23	5	513631	295438	1039445	352589
168	5	568825	437556	1050500	3857
6633	6	1403428	711313	2752778	931222

3.2 Column Apparatus

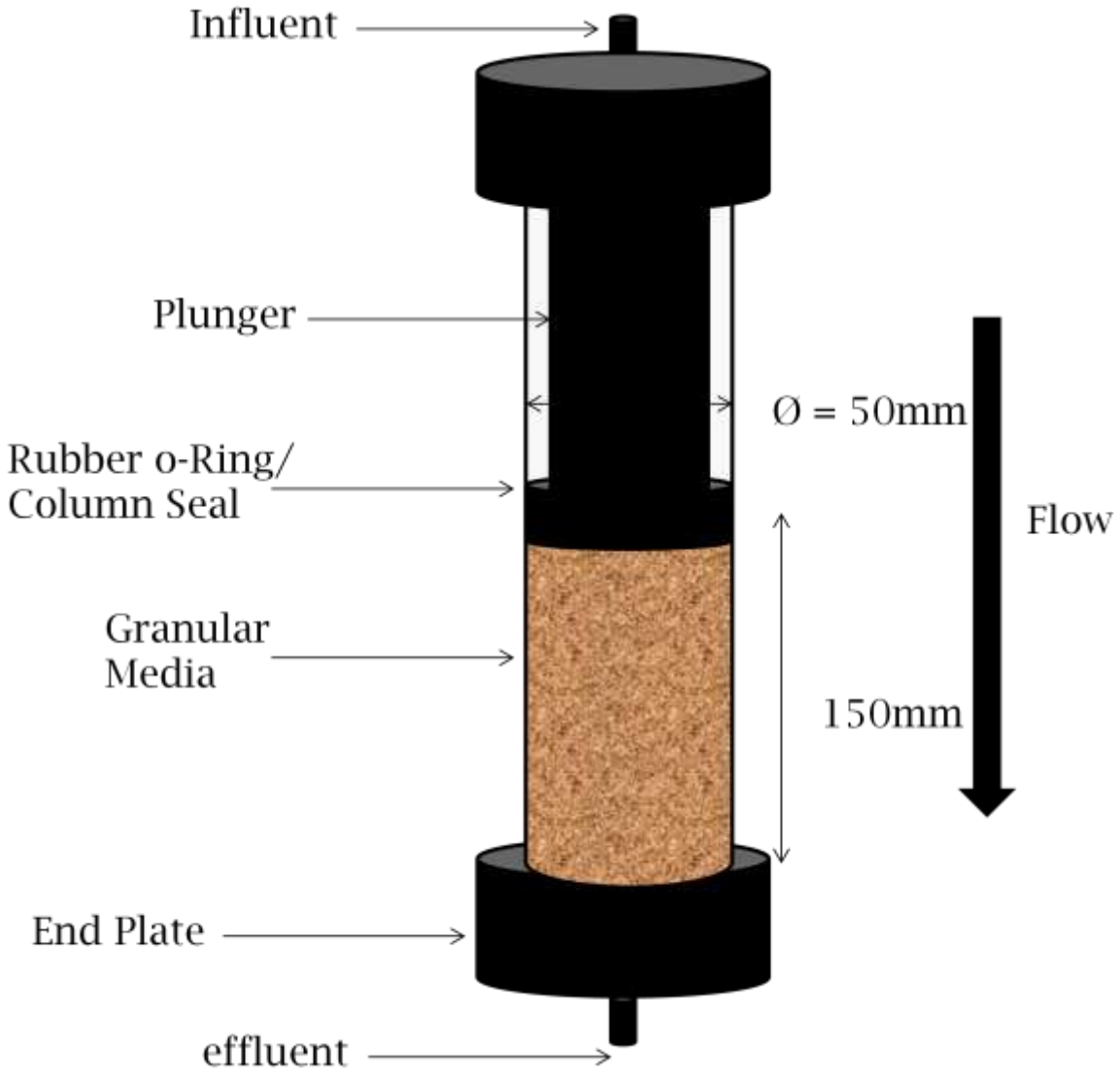

Figure 3.2 Bench-Scale Column

The columns (labelled A and B) used in this investigation (Spectra/Chrom® Aqueous Columns, Spectrum Chromatography ®, Houston, TX), had an internal diameter (Ø) of 50mm and a length of 300mm, and were packed with media to a depth of 150-160mm. Each borosilicate glass column was fitted with upper and lower polypropylene end plates (Figure 3.3). A fine mesh support (4"x 4") prevented media from entering the effluent portal on the lower endplate. Above the 150mm of packed media, a plunger and Viton®O-ring were used to pressure-seal the column by expanding the O-ring against the inner column wall. Between each experiment, the glass columns were rinsed with bleach, soaked overnight and/or autoclaved, then rinsed with ethanol and sterile water.

3.2.1 Column Packing

Columns were packed with new media for each trial. All materials used to pack the column apparatus were sterilized and a Bunsen burner was lit in the vicinity of the columns for the duration of packing. Each column was packed with evenly mixed, saturated and autoclaved media to a depth of approximately 150mm (±5mm). The procedure is summarized as follows:

1. Completely seal column at effluent portal
2. Add sterile water to a depth of 30mm
3. Add sand-water slurry in 20mm increments
4. Agitate slurry to remove air and ensure complete settling by applying a handheld massager (Hitachi Magic Wand, Hitachi Limited, Ontario) to the outer wall of column
5. Repeat steps three and four until 150mm of media have been added and compacted
6. Do not allow water level to drop below media level at any time during packing
7. Seal column using O-ring and plunger
8. Initiate flow

3.2.2 Column Operation

Columns A and B were oriented vertically (Figure 3.2) and operated in down-flow mode. Both columns were fed with a singular feed suspension using Masterflex Chem-Durance® L/S Bio-Tubing. Specifically, *B. subtilis* spores or MS were fed into the columns using a Cole-Parmer Masterflex® L/S ® Digital Standard Drive Easy-Load

peristaltic pump (Cole-Parmer Instruments Co., Montreal, Québec) with three pump heads. Each column had a dedicated pump head for the *B. subtilis* feed suspension, and one pump head was used for rinse solutions. After packing, columns were flushed with one pore volume (PV) of sterile deionized (DI) water prior to the introduction of the bromide (Br^-) tracer. Prior to the introduction of colloids, the columns were flushed overnight with a minimum of ten PVs of sterile, colloid-free AGW background solution to standardize the chemical conditions in the column (Knappett et al., 2008).

Colloid seed suspensions were continually mixed with a submerged stir bar, and introduced into the columns at a flow rate of 1.2mL/min (±0.4mL/min) to obtain a loading rate or approximately 0.1m/hour. Flow was measured at the effluent portal using a graduated cylinder after the collection of each colloid sample (Appendix B). Seed suspensions were fed continuously into the columns for approximately 5 PVs (about 8 hours; it was estimated that experiments of this length would reach a pseudo steady-state of colloid breakthrough beginning at ~2 PVs or about 180 minutes). Once the final sample was collected, the pressure was released from the columns, allowing them to drain and they were emptied for washing and sterilization.

3.3 Tracer Tests and Feed Suspensions

3.3.1 Solute Tracer Tests

Conservative tracer tests using a concentrated bromide solution (200mg/L; 2.5mM, Fischer Scientific, Fairlawn, NJ) were performed each time the column apparatus was packed to identify any gross packing problems, confirm one PV (~119mL; Appendix A) and estimate time to colloid breakthrough to ensure appropriate sampling schedules. Reproducibility in solute breakthrough curves should ensure experimental repeats are not affected by variability in column packing. A bromide (Br^-) tracer solution was prepared by dissolving 200mg in DI water and autoclaving at 121°C for a minimum of 15 minutes. The sterile feed solution was pumped into both columns simultaneously at a flow rate of 1.2mL per minute for approximately 160 to 180 minutes or 2 PVs. Once breakthrough was sustained a rinse solution was introduced using the appropriate AGW). Effluent samples were collected using glass test tubes and analyzed using a HACH C0150 conductivity meter with standard probe 50161; to minimize evaporation measurements were performed after initiation of the rinse

solution (APHA 2510, 1998). The Br^- solute breakthrough curves (Appendix B) were used to modify (bio)colloid sampling schedules to increase the accuracy of breakthrough estimates. Breakthrough was anticipated at 90 minutes (pore volume calculation, appendix A) but (bio)colloid sampling was moved by increments of five or ten minutes (ie. sampling at 80 or 85 minutes) if the Br^- solute breakthrough differed from 90 minutes.

3.3.2 Dye Tracer

Brilliant blue FCF dye (4 grams/L; Colour Index 42090) was used to stain flow pathways, allowing a visual assessment to determine if ideal plug flow occurred in the columns or preferential pathways developed (German-Heins and Flury, 2000; Passmore et al., 2010). The dye suspension was pumped into the columns using the same flow rate used in all experiments (1.2mL/minute). This tracer test was preformed after one experiment conducted with *Bacillus subtilis* spores.

3.3.3 Colloid Feed Suspensions

Colloid feed suspensions were prepared by adding solid potassium chloride (KCl) to sterilized DI water in glass media bottles (Gannon et al., 1991; Bradford et al., 2002; Bradford et al., 2004; Kim et al., 2008a). KCl is often used as a background electrolyte solution (Castro and Tufenkji, 2007). The DI water had a conductivity of 0.1µS, a dissolved oxygen concentration of 6.81at 24.5°C, and a pH of 5.86 (HACH C0150 conductivity meter, standard probe 50161, APHA 2510, 1998). The ionic strengths used were 0.1mM KCl and 10mM KCl. *B. subtilis* spores were exposed to the AGW solutions for 14hours at 4°C with constant stirring prior to passage through the columns. Each feed suspension was created with a target total concentration of $1.0x10^6$colloids/mL; each experiment used only one colloid. Small aliquots of the purified spore suspensions, or MS preparations were added to the AGW solution as required to obtain a calculated feed concentration of $1.0x10^6$ CFU/mL or MS/mL. To verify actual colloid concentrations in each seed suspension, five samples of 2.5mL were drawn from the feed suspension at the beginning and end of each experiment (10 samples total) and enumerated in triplicate. To assess the impact of the overnight storage in 10mM KCl, samples were collected immediately after inoculating the AGW solution for some experiments and after 14 hours exposure to compare concentration and quantify any losses (5 samples).

Spore suspensions were transferred to room temperature immediately prior to initiating each experiment; storage at 4°C prevents spore germination (ASTM, 2011; Kim et al., 2008a). Colloid suspensions remained at room temperature for the duration of the experiment with constant stirring. A large, submerged stir bar was continually moving at a manual setting of 300 rotations per minute (RPM) (VWR International, Radnar, PA).

3.3.4 Feed Suspension Spore Concentration Distribution

To assess the efficacy of stirring and the distribution of *B. subtilis* spores in the feedstock suspension, a stock suspension of strain 6633 was prepared according to the methods described above. Eighteen samples were withdrawn at six depths along the outer edge of the media bottle, the middle of the liquid radius, and the centre of the vortex (Figure 3.3). These samples were diluted as necessary and plated in triplicate. This stock suspension was autoclaved, then discarded.

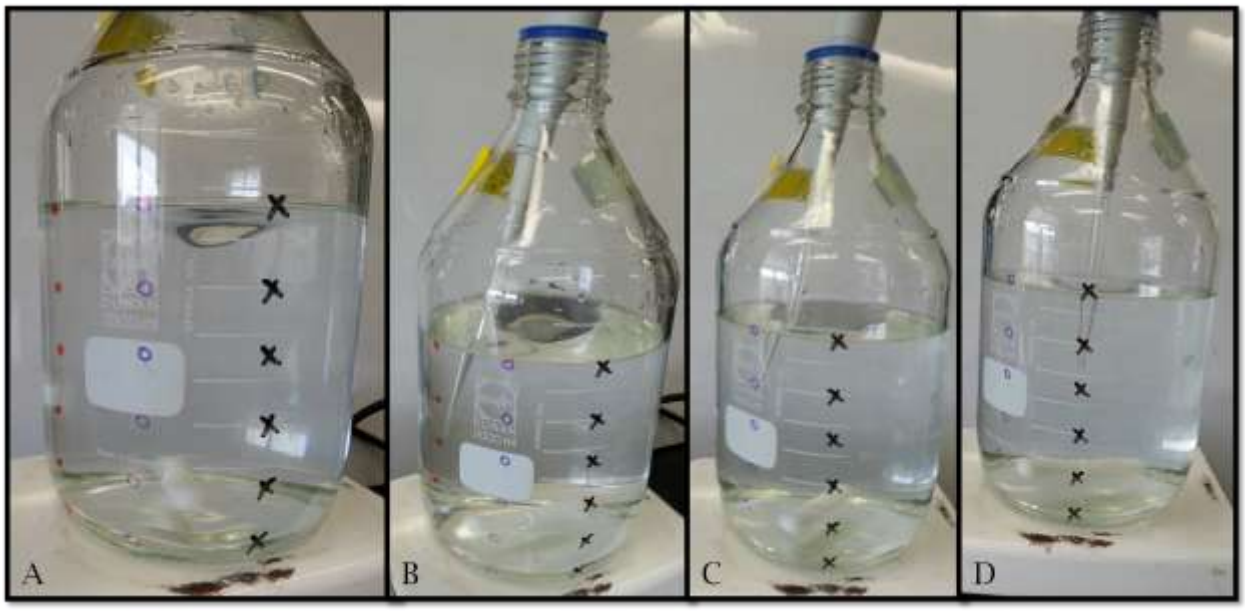

Figure 3.3 Feed Suspension Spore Concentration Distribution Assessment (A. Collection Point Labels, B. Sample 03, C. Sample M2, C. Sample C3)

3.4 Granular Media Selection and Characterization

High purity silica sand (99.5% SiO_2, Best Sand Corporation, Chardon, Ohio) was selected for this study to represent a granular media typical in riverbeds and shallow aquifer systems (D_{50} ranging from 0.2 to 1.25mm, UC ~ 1.9 (Harvey et al., 1995; Pang et al., 1998; Côté, 2004) (Table 3.2, Figure 3.3). Quartz grains are sub-rounded and irregular

in shape (Castro and Tukenkji, 2007) with heterogeneous mineralogy (Best Sand, 2013). Well-sorted, angular alluvial sands or silty sands of this size might be encountered near an extraction well used in RBF (Pang et al., 2005; Knappett et al., 2008).

Table 3.4 Typical Chemical Analysis of Best Sand (pH = 7) (Best Sand, 2013)

Component	Silica Sand Content
SiO_2	99.5 %
Al_2O_3	0.147 %
TiO_2	0.031%
Fe_2O_3	0.092 %
CaO	0.006 %
LOl	0.12 %
Traces	NaO_2 MgO K_2O FiO

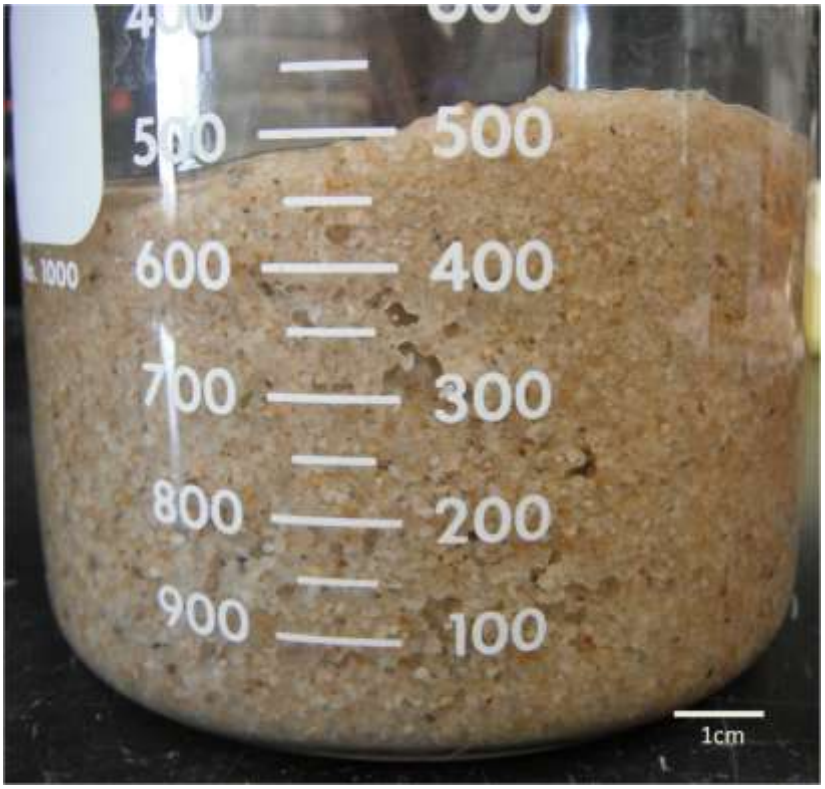

Figure 3.4 Quartz Sand Employed in Column Studies (in a 1000mL beaker)

For this investigation, samples of quartz sand were sieved to achieve a median grain size (D_{50}) ranging from 0.83 to 0.93 and a uniformity coefficient (UC) ranging from 1.81 to 2.02 with an effective size (ES) of ~0.50 (Appendix A). To prepare the media for each experiment, pre-sieved, measured samples were wetted and autoclaved at 121°C for 15 minutes to sterilize the material and provide percolation to remove air pockets. Sieving was performed using a known mass of well mixed sand which was agitated mechanically through a set of sieves (ASTM, 2001). The percent mass remaining on each mesh size was recorded and the cumulative weight by mesh size plotted. The effective size (D_{10}), uniformity coefficient (D_{60}/ D_{10}), and D_{50} were then calculated from these data. Grain size analysis was completed for a bulk batch of media used during experiments one through five, and prior to each of experiments six through 22. To summarize, the procedure is:

1. Record the mass of each sieve (grams), including the solid sieve pan
2. Arrange sieve pans according to mesh size with the smallest mesh at the bottom
3. Weigh a small volume of sand (100g to 800g)
4. Pour into the top sieve and initiate physical agitation
5. Set timer for 10 to 20 minutes

6. Weigh each sieve with sand contents
7. Subtract the pan weight to determine cumulative mass remaining in each mesh size

The porosity of the quartz sand was determined using a 100mL graduated cylinder (GC) in a wet-dry cycle according to the following procedure:

1. Record mass of the empty cylinder (A)
2. Record mass of the cylinder filled to 100mL with water (B)
3. Record the mass of the cylinder filled with wet-packed media and agitated with a physical massager to increase settling and remove air (D)
4. Dry the cylinder for 24 hours in a dessicator at approximately 115°C until all water has evaporated and record the weight of the cylinder filled with dry sand (C)

Use equation 3.1 to calculate the porosity of the sample based on the ratio of void space (V_v) to the bulk volume of the material (V_t).

$$Porosity = \frac{V_v}{V_t} \qquad \text{equation 3.1}$$

Where:

$$\frac{V_v}{V_t} = \frac{(D - C)}{(B - A)} = \frac{(mass\ of\ wet - packed\ media\ in\ GC) - (mass\ of\ dried\ media\ in\ GC)}{(mass\ of\ water\ in\ GC) - (mass\ of\ GC)}$$

This equation assumes that appropriate packing procedures will ensure all voids are filled with water. The porosity of the media used ranged from 0.36 to 0.41, or 36-41% (Appendix A).

3.4.1 Spore Concentration Distribution in Column Media

To assess the distribution of colloids retained in the column media, samples of approximately 2.5 cm depth were collected after Trail #22 from Experiment 6 (6633-6). At the completion of the trial, the columns were depressurized and allowed to drain slowly to avoid shear forces from causing colloid sloughing. When the columns were emptied of liquid the bottom endplate was carefully removed and samples of 80 to 120 grams collected. Each sample was transferred into a sterile container, mixed with 20mL of the appropriate background solution and sonicated for three minutes

(Passmore et al., 2010). Sonication is expected to disperse colloids with minimal damage to cellular membranes. A 2.5mL aliquot of fluid was withdrawn immediately after sonication and plated in triplicate to assess the distribution of spores throughout the column media.

3.5 Colloid Selection, Preparation and Enumeration

3.5.1 *Bacillus subtilis*

B. subtilis spores were used in all column experiments with the exception of Experiment 1 and 16, which used microspheres (MS) only. Four strains of *B. subtilis* were used to represent lab-type and wild-type strains.

3.5.1.1 Strain Selection

Two well-studied and commonly used laboratory-type (lab-type, LT) strains and two wild-type (WT) strains were selected for these experiments (Figure 3.4). *Bacillus subtilis subtilis* strain 168 (BGSC 1A1) is well characterized and considered a classic example of *Bacillus subtilis;* it is the most studied strain of the species (Daniel Zeigler, personal communication). The historical WT parent of 168 is strain 3610 from the National Collection of Industrial Bacteria (NCIB, Edinburgh, Scotland). Strain 3610 produces biofilms and structured colonies, whereas strain 168 does not (Zeigler 2008). These two represent the subspecies *subtilis* as WT and LT *Bacillus subtilis.*

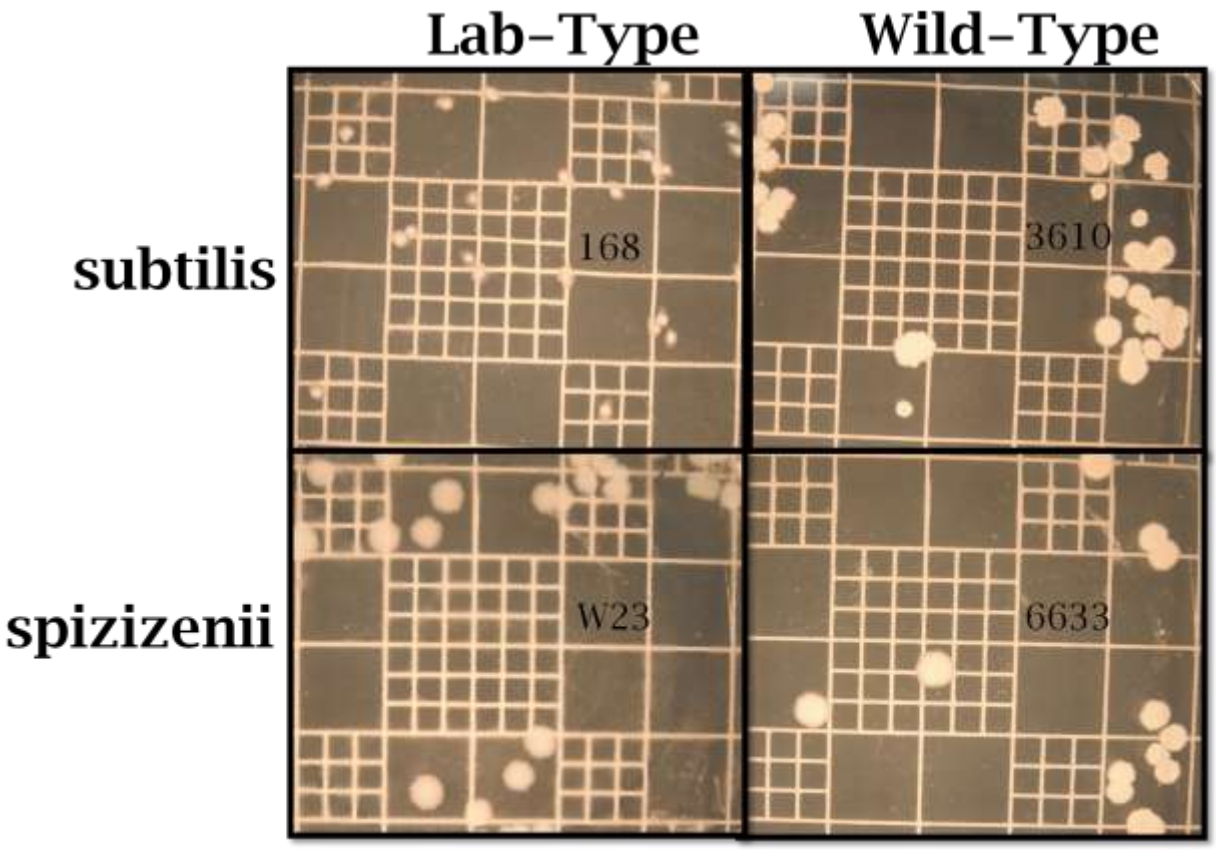

Figure 3.5 *Bacillus subtilis* subspecies and strains used for this Investigation

To select a second pair for comparison, several strains were examined. Strain NCIB 8649 (*Bacillus subtilis* variety niger) has been used in several lab and field trials (Sinton et al., 2000; Chik et al., 2012); it is able to produce readily-visible orange colonies, making it an easily enumerated strain (Houston et al., 1989). It was used in field investigations of pathogen removal via riverbank filtration in 2009 and an obvious choice for this study. However, it was reclassified as *Bacillus atrophaeus* (BGSC 12A1) after genetic analysis by PCR (Fritz and Pukall, 2001). As more complete genetic analyses are performed, it is possible that other strains currently classified as *Bacillus subtilis* and used in performance demonstrations may be reclassified. A separate subspecies was desirable to provide some contrast to the classic *Bacillus* 168 with definite genetic differences.

Subspecies *spizizenii* was selected because it should be genetically less similar to 168 than the previously used 8649 (Zeigler, personal communication). The two subspecies *subtilis* and *spizizenii* differ significantly in cell wall composition (Daniel Zeigler,

personal communication). The domesticated version, strain W23, does not swarm or form fruiting bodies when grown on laboratory media. The WT parent of W23 is ATCC 6633. The genome of W23 differs from the sequence of 6633 by approximately one hundred bases. By selecting very genetically different subspecies, the transport behavior of any spore-forming aerobic bacteria of the *Bacillus* species encountered in the environment should fall within the range encompassed in this study.

Table 3.5 Strains of *Bacillus subtilis* used in this study

Strain Type	Full Name	Accession Number / external reference name	Call Code in this study
Laboratory	*Bacillus subtilis subtilis* strain 168	BGSC 1A1	168
	Bacillus subtilis spizizenii strain W23	BGSC 2A9	W23
Wild	*Bacillus subtilis subtilis* strain 3610	NCIB 3610 BGSC 3A1	3610
	Bacillus subtilis spizizenii strain 6633	ATCC 6633	6633

3.5.1.2 Propagation

Pure Cultures of each *Bacillus* strain were obtained by single-colony isolation via streaking vegetative cultures onto nutrient agar plates (Figure 3.4).

Figure 3.6 Single-Colony Isolation of LT 168 and LT W23 via streak-plating of vegetative cells

Pure-culture spores were then derived from vegetative cells according to the nutrient exhaustion approach outlined by Nicohlson and Setlow (1990), using 2xSG agar and the following procedure:

1. Flood 15cm diameter solid agar Petri dishes with 3mL portions of pure *B. subtilis* culture in nutrient broth (each Petri dish should contain 30mL solid media)
2. Incubate plates upright for 10 – 14 days, or until 80-90% sporulation is achieved (verified by Schaeffer-Fulton staining method) (Mormak and Casida, 1985; Hussey and Zayaitz, 2007)
3. Flood Petri dishes with 20mL of 1x PBS and allow to rest for at least 15 minutes
4. Using a sterile glass or plastic rod, gently scrape the surface of the agar to collect superficial growth
5. Using a sterile funnel transfer the contents to a sterile centrifuge tube
6. Purify suspension as per ASTM standard E2111-00 (ASTM, 2000).

3.5.1.3 Purification

B. subtilis spores were purified following ASTM standard E2111-00 (ASTM, 2000). This involved centrifuging spore suspensions at 10,000g for 10 minutes at 4°C using a 21000R centrifuge, pouring off the supernatant and resuspending the pellet in 20mL of sterile DI water by vortexing for 20 seconds. This procedure was repeated three

times to ensure a high purity spore suspension with a high concentration of colony forming units (CFU). Suspensions were refrigerated at 4°C until use.

3.5.1.4 Enumeration

B. subtilis spores were enumerated by spread plating on nutrient agar (Standard Methods, 2004; ASTM, 2011). All effluent samples collected were heatshocked prior to the preparation of serial dilutions in a hot bath at 80°C for ten minutes. Serial dilutions were prepared with 0.1% Tween 80 in sterile DI water to decrease aggregation (Cartier et al., 2007; Kim et al., 2008a). Dilutions were vortexed for a minimum of ten seconds to homogenize the sample prior to transferring 0.1 to 0.5mL of the dilution onto the surface of a dried agar plate. Drying plates for 30 minutes prior to spreading reduces colony spreading, and increases counting accuracy (Francis et al., 2001). Typically triplicate samples of 0.1 or 0.2mL were used to enumerate effluent samples. Plates were inverted and incubated at 30°C for 22-27 hours. Spores were identified by their white colony growth (Figure 3.7). For *B. subtilis,* a target range of 30 to 300 CFUs per plate was targeted (Emelko et al., 2008) and used to calculate the number counted per volume processed according to the following general equation:

$$Concentration\left(\frac{CFUs}{plate}\right) = \frac{mean\ of\ plate\ counts}{volume\ filtered\ (mL)}\ x\ dilution\ factor \qquad \text{equation 3.2}$$

B. subtilis removals ($\log_{10}$) were calculated by subtracting the $\log_{10}$ of the filter effluent concentration from the $\log_{10}$ of the influent concentration. Percent removals were calculated by subtracting the effluent concentration (C) from the stock concentration (C_0) and dividing this total by the stock concentration (C) before multiplying by 100 (to produce a %).

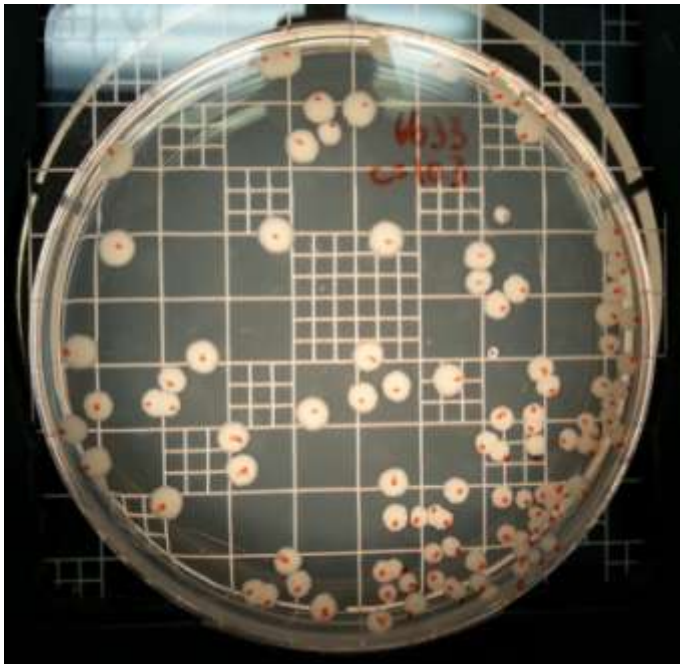

Figure 3.7 Colony Growth on Nutrient Agar after 24 hours incubation

3.5.2 Microspheres

Two sizes of surfactant-free, carboxylate-modified fluorescent-dyed polystyrene microspheres were used as non-biological bacterial- and protozoan-size surrogates (1.1µm and 4.5µm, respectively). The proprietary dyes allow the use of fluorescence microscopy, show low photobleaching, and are hydrophobic so they will not partition into the aqueous phase during experimentation. The Nile Red (NR) microspheres (Fluoresbrite™, Polysciences Inc., Warrington, PA) were provided in a 2.0% suspension of aqueous solids with an average nominal diameter of 1.0µm, a density of 1.05g/mL, a maximum excitation at 535nm and a maximum emission at 575nm. The yellow-green (YG) microspheres (Fluospheres®, Invitrogen Corporation, Grand Island, NY) are packaged as a 2.63% aqueous suspension of 4.99×10^8 particles/mL with a coefficient of variation for the MS diameter of 7% and a density of 1.05g/mL. The YG produced a maximum excitation of 441nm and an emission maximum of 486nm. The Material Safety Data Sheets provided by the manufacturers indicated that no hazardous components were contained in the MS products. No biocides or stabilizers are added to the MS and they are packaged in DI water.

3.5.3 Preparation

The MS were stored at 4°C in the dark prior to suspension in the appropriate background AGW solution. The weight to volume packaging allowed for the calculation of the particle concentration per millilitre (mL) according to the following equation (Invitrogen Corporation, 2013):

$$number\ of\ particles\ per\ mL = \frac{6C\ x\ 10^{12}}{p\ x\ \pi\ x\emptyset^3} \qquad \text{equation 3.3}$$

Where:

C = concentration of suspended beads in g/mL (0.02 g/mL for 2% solids)

Ø = diameter in microns of MS

and

ρ =density of polymer in grams per mL (1.05 g/mL for polystyrene)

The manufacturer's weight to volume method (equation 3.3) was used to determine the volume of stock microsphere suspensions (both NR and YG) that was added to the feedstock suspensions during seeding experiments.

3.5.4 Enumeration

To calculate effluent microsphere concentrations, samples of approximately 2.5mL were collected at the effluent portal, and serial dilutions prepared as necessary using 9.0mL sterile DI water. Each sample was thoroughly vortexed for 10 seconds prior to dilution. Diluted MS were concentrated and enumerated using fluorescence microscopy of membrane filters at 100x magnification and 200x magnification for the YG and NR MS respectively, using a direct-count method (Emelko et al., 2003). In brief, this method involves filtering aliquots of 0.1 to 8mL through a 0.4µm nominal porosity polycarbonate 25mm diameter membrane (Nuclepore filter membrane, Whatman Inc., Florham Park, NJ) resting on a 8.0µm nominal porosity nitrocellulose membrane (SCWP 02500, Millipore, Billerica, MA) using a vacuum pump. The polycarbonate filter was then mounted on a glass slide using DABCO in glycerol as a mounting medium and each slide was counted manually using an AXIOSKOP 2 Plus microscope (Zeiss, Oberkochen, Germany). All microspheres on the slides were enumerated by scanning

the entire filter area. Counts between 30 and 300 spheres/slide were targeted (Emelko et al., 2008).

3.5.5 Zeta Potential and Size Analysis

The size of each colloids was evaluated using dynamic light scattering (DLS) (Zetasizer Nano Series, Malvern Instruments Ltd., Worcestershire). Triplicate measurements were obtained using 10 second intervals to determine the equivalent spherical diameter.

The zeta potential (mV) of the six colloids, measured as electrophoretic mobility, was determined using clear, disposable polycarbonate capillary cells (DTS1061, Malvern Instruments Ltd., Worcestershire). A combination of the electrophoresis and laser Doppler velocimetry techniques measured how fast the particles moved in their background solutions when the electric field was applied and calculated the resulting potential at the slipping plane using Smoluchowski's equation (Zetasizer Nano Series, Malvern Instruments Ltd, Worcestershire). Triplicate measurements were taken at 20°C, and several at 4°C using concentrated colloid suspensions (10^7 to 10^9 MS or CFU/mL).

3.6 Data Analysis

Bacillus subtilis and MS data were examined using effluent concentration breakthrough curves and/or box-and-whisker plots. Adjusted breakthrough curves (BTCs) plotted as normalized concentrations over time (C/C_0) and log removals over time ($\log_{10} C/C_0$) were used to determine when pseudo steady-state spore and MS removal occurred in the saturated porous media (the plateau):

Where:

C = concentration of colloids in a sample collected at the effluent portal in colloids/mL

And:

C_0 = concentration of colloids in the feed suspension as calculated using samples collected pre- and post-experiment in colloid/mL

The BTCs show variability in the time to breakthrough (T_B) for each experiment (colloid type) and trial (experimental repeats) and the time for the column effluent to reach a pseudo-steadystate plateau. Five influent/effluent colloid concentration pairs from the

final five samples collected from each trial were utilized in calculating pseudo steady-state colloid percent removal. Box-and-whisker plots, comparing the distribution for each colloid were used. The bottom and top of the box are the 25th and 75 percentile respectively, while the bar generally near the centre of the box is the median colloid removal (50th percentile). The whiskers indicate 1.5 times the interquartile (IQ) range with extreme labels, falling outside the 1.5xIQ range labeled with an asterisk (*). The IQ is defined as the difference between the 75th and 25th percentile.

The arithmetic means for each experiment and strain were compared using Analysis of Variance (ANOVA). Univariate statistical ANOVA analysies were performed using IBM Statistical Product and Service Solutions Statistics (SPSS) (IBM Corporation, 2013) to investigate if there were any statistical differences between the means of percent colloid removal. Levenes' test was performed to evaluate homogeneity of variances prior to the ANOVA analysis. Single factor ANOVA was used to determine if there were significant differences in *Bacillus subtilis* recovery from the two water matrices (0.1mM and 10mM KCl), and if the differences between strain recovery in each water matrix were significant and if any interaction between strain and type of *Bacillus subtilis* occurred. The use of a univariate ANOVA allows causal relationships between independent and dependent variables to be identified. In the cases mentioned above, the independent variable (strain, type or water matrix) is manipulated while the dependent variable (log removal) is held constant. The analyses were performed using a significance level (α) of 0.05.

Chapter 4 Results and Discussion

4.0 Introduction

The objective of this investigation was to assess if lab-type (LT) and wild-type (WT) strains of *B. subtilis* would show substantially different ($p < 0.05$) mean removal during transport through bench-scale granular media filtration columns, and to contrast spore removal to removal of non surface-active polystyrene MS. Two subspecies of *Bacillus subtilis* were used, and each subspecies had a LT and WT strain. Two sizes of MS were used, 1.1µm and 4.5 µm, as bacterial and protozoan-sized pathogen surrogates respectively. Twenty-two Trials within Six Experiments were conducted using duplicate columns operated at a loading rate of 0.1m/hour, an ES of ~0.5mm silica sand and ionic strengths of 0.10mM and 10mM KCl. The results include duplicate tracer breakthrough curves, (bio)colloid breakthrough curves at the two ionic strength conditions, and pseudo stead-state (bio)colloid removal. A one-way ANOVA was conducted to analyze the differences between group means for strain, subspecies, and type. Sample handling errors were assessed to determine the effects uncontrolled factors might have had on the $\log_{10}$ removal calculations. Size and zeta potential measurements of all (bio)colloids was also assessed.

4.1 (Bio)colloid Characteristics

4.1.1 Zeta Potential

To accurately measure (bio)colloid electrophoretic mobility and size using the DTS Zetasizer Nano, a milky sample is recommended by the maufacturer (Malvern, 2004). To achieve an adequately concentrated suspension a spore concentration of ~10^9 CFU/mL was required. The electrophoretic mobility of each (bio)colloid was measured before and after 14 hours of exposure to the AGW solutions and in DI water with Tween80 (0.1%), as used for serial dilutions (Table 4.1). All measurements were conducted using previously grown *Bacillus subtilis* stocks. Consistent with other reports, all (bio)colloids used in this study were negatively charged at the conditions examined (Sharma et al., 1985; Gannon et al., 1991; Rice et al., 1996; Ahimou et al., 2001; Bradford et al., 2004, 2007; Schinner et al., 2010). Exposure time did not significantly affect the measured zeta potentials.

The zeta potential of *Bacillus subtilis* strains 3610, W23, and 6633 at the three conditions used during the course of this investigation (serial dilution suspension: DI +Tween80; Background AGW solutions: 0.10mM and 10mM KCl) ranged from -30 to -43mV at neutral pH (SD 4-6mV). Zeta potential remained stable in low ionic strength solutions (DI + Tween80 and 0.10mM KCl), regardless of exposure time (Table 4.1). Because a small change was observed between the zeta potential of spores suspended in DI+Tween80 and spores suspended in the two AGWs used for the feed stock spore suspensions, future studies should endeavour to use AGW for serial dilutions. In high ionic strength solution (10mM KCl) spore zeta potential became more negative initially (-41.6mV, -39.9mV, and -43.1mV), but became slightly less negative after 14 hours of exposure (-37.5 mV, -38.8mV, -41.4mV).

Table 4.1 Zeta Potential of (Bio)Colloids at Experimental Conditions

			NR MS 1.1		YG MS 4.5		3610		W23		168		6633	
			ZP	SD	ZP	SD	ZP	SD	ZP	SD	ZP	SD	ZP	SD
Conditioning Solution:	**Exposure Time (hours):**	**T (°C):**												
DI+Tween80	0.5	25	/	/	/	/	-31.8	4.1	-33.1	4.9	-50.7	4.8	-32.9	4.5
0.1mM	0.5	25	-47.3	3.8	-67.8	4.9	-35.4	4.8	-33.6	6.4	-58.5	4.7	-32.9	3.9
	14	25	-46.2	4.6	-66.3	3.8	-29.3	3.9	-33.6	3.9	-62.1	3.6	-31.9	4.2
		4	/	/	/	/	-43.3	4.9	/	/	-46.1	4.5	-45.7	6.6
10mM	0.5	25	-60.6	4.9	-86.7	5.3	-41.6	5.2	-39.9	4.7	-65.4	4.6	-43.1	5.4
	14	25	-58.7	5.4	-86.4	6.9	-37.5	6.2	-38.8	6.3	-55.8	5.4	-41.4	5.6

Bacillus subtils subtilis LT strain 168 registered a more negative zeta potential than the other three strains. In DI water, strain 168 measured -50.6mV, becoming more negatively charged with increasing ionic strength. Unlike the other strains, exposure to 0.1mM KCl increased the negativity of zeta potential. All spores were suspended in the same background solution stock, confirming the findings of Ahimou et al. (2001), who measured the zeta potential of three *Bacillus subtilis* spore strains and found appreciable differences between different strains (ATCC 7058, 12432, 15811). At neutral pH, the three strains measured approximately -10, -20, and -45mV respectively (Ahimou et al., 2001). Zeta potential is specific to each bacterial strain. The zeta potential of the four strains used in this study were most similar at the lower temperature of 4°C (~44mV in 0.1mM KCl). Three strains became less negative with increasing temperature, while strain 168 became more negative as the temperature changed to 25°C. These results indicate that changes in the zeta potential of bacterial

spores from the same species could impact attenuation in saturated porous media, in agreement with the findings of Ahimou et al. (2001).

The results presented here are suspect because they contradict both theory and other investigations (Sharma et al., 1987; Gannon et al., 1991; Ongerth 1996; Lytle et al., 2002; Dai and Hozalski, 2003; Kuznar and Elimelech, 2004). A study by Rice et al. (1996), found that zeta potential became less negatively charged at higher ionic strengths in accordance with CFT; an increasing ionic strength provides a larger number of ions in solution to compress the electrical double layer associated with a colloid's surface. Other studies hypothesized that at higher ionic strengths bacterial EPS and surface appendages would coil and remain close to cell walls, therefore becoming less negatively charged at the cell surface (Camesano and Abu-Lail 2002; Frank and Belfort, 2003; Kuznar and Elimelech, 2004). Three of the strains studied here showed the opposite trend, becoming more negatively charged in the higher ionic strength solution (10mM KCl). An investigation of surface appendages for motile *Bacillus* strains is recommended. Because these results contradict theory as well as the results of other investigations, a reanalysis was performed (Table 4.2).

Table 4.2 Zeta Potential of *Bacillus subtilis* in AGW (mV)

	3610		**W23**		**168**		**6633**	
	ZP	SD	ZP	SD	ZP	SD	ZP	SD
AGW								
0.1mM	-31.1	8.6	-34.8	5.5	-66.7	4.6	-35.9	4.9
	-33.8	9.8	-35.3	5.8	-66.0	5.9	-34.9	4.4
	-33.1	10.4	-34.0	5.9	-66.7	4.6	-35.0	4.5
10mM	-36.9	5.2	-37.7	7.1	-53.1	5.3	-41.2	5.2
	-38.7	5.3	-33.5	7.6	-57.2	5.0	-44.3	6.1
	-40.4	5.5	-39.0	6.8	-58.4	5.7	-44.7	5.6

*all measurements performed at 25°C

These results are unexpected and show significant differences between the mean values for the zeta potential of the *Bacillus* strains at the two IS used as AGW ($p<0.05$) except for *Bacillus subtilis subtilis* LT 168. Laboratory strains have been genetically modified and adapted for use in experimentation suggesting that further investigation into the variability of WT strains is required. These results could be erroneous, caused by errors with machinery, improper use of equipment, failure to calibrate equipment,

inappropriate preparation of samples, etc. They could also be caused by the aging of spore suspensions that were held in storage at 4°C for several months. The data presented here disagree with fundamental theory, and experimental findings reported elsewhere, and represent only the spore suspensions analyzed. Further investigation into the zeta potential of fresh spore suspensions comparing a variety of *Bacillus* strains is required to validate these findings.

Table 4.3 Analysis of Variance of Spore Zeta Potential (mV) by Ionic Strength (mM)

(Bio)Colloid		Sum of Squares	df	Mean Square	F	Sig.
1.1 NR MS	Between Groups	166.410	1	166.410	138.100	.007
	Within Groups	2.410	2	1.205		
4.5 YG MS	Between Groups	380.250	1	380.250	650.000	.002
	Within Groups	1.170	2	.585		
3610	Between Groups	104.976	1	104.976	22.031	.002
	Within Groups	38.120	8	4.765		
W 23	Between Groups	30.976	1	30.976	8.959	.017
	Within Groups	27.660	8	3.458		
168	Between Groups	91.204	1	91.204	5.316	.050
	Within Groups	137.256	8	17.157		
6633	Between Groups	194.481	1	194.481	72.908	.000
	Within Groups	21.340	8	2.668		

*5% significance detected where $p < 0.05$

Bradford et al. (2007), measured the electrophoretic mobility of carboxyl latex colloids as zeta potentials and found no change over a range of 6 to 56 mM NaCl. Once the ionic strength reached 81 mM, ZP moved from -104 mV to -78 mV. This could suggest that smaller changes in ionic strength do not, or have less of an impact, on the electrophoretic mobility of some (bio)colloids.

The zeta potential of the large YG MS was -67.8mV and -86.7mV in 0.10mM and 10mM KCl respectively, in agreement with other findings (Bradford et al., 2007; 2004; Gannon et al., 1991; Sharma et al., 1985), with no substantial change after 14 hours exposure to the AGW, which was expected for non-living particles. The NR 1.1µm MS were less negatively charged, measuring -47.3mV and -60.6mV at 0.10mM and 10mM respectively.

4.1.2 Size

The size measurements from DLS analysis (Z-average) of the (bio)colloids confirm the bacterial and protozoan-sized MS were approximately 1 and 5 µm respectively. The *Bacillus subtilis* spores ranged in size from 1.9 to 2.9µm (Table 4.2).

Table 4.4 (Bio)Colloid Size Measurements (DLS) in Experimental Solutions

	(Bio)Colloid Size(µm) at 25°C											
	NR MS 1.1		YG MS 4.5		3610		W23		168		6633	
	Size	SD	Size	SD	Size	SD	Size	SD	Size	SD	Size	SD
Conditioning Solution:												
DI+Tween80	/	/	/	/	2.441	0.073	2.463	0.135	2.124	0.034	2.727	0.107
0.1mM	/	/	4.976	0.742	2.340	0.237	2.678	0.053	2.331	0.120	/	/
10mM	1.020	0.090	5.199	n/a	2.072	0.134	2.589	0.105	1.940	0.220	2.963	0.119

According to CFT, the size of the bacterial spores fall in the range of the minimum contact efficiency (0.5 - 3µm) (Yao et al., 1971) and these measurements suggest that the spores should be removed to a lesser extent than the larger, protozoan-sized colloids (4.5µm) used in this investigation.

4.2 Tracer Tests

4.2.1 Solute Tracer Test

Flow of feed suspensions into the columns ranged between ~1.1 and ~1.4mL/minute, as measured by graduated cylinder at the effluent portal after the collection of each sample (Table 4.3). The accuracy of flow measurements by graduated cylinder was confirmed by weighing the volume of filter effluent collected over a one minute period after the collection of each sample during Trial #22 (6633-6) and comparing that to the flow as measured by volume (Table 4.4). Identical tubing configuration and set-up was employed with stacked pump heads on one pump drive; flow measurements were

consistently higher for Column A (~0.1-0.2mL/min; Table 4.4) potentially contributing to earlier breakthrough of bromide and colloids in Column A.

Table 4.5 Flow Rate Measurements (Trial #8: 3610-2)

Sample Number	Elapsed Time (minutes)	Flow Column A (mL/min)	Flow Column B (mL/min)
0	0	1.4	1.3
1	30	1.2	1.2
2	60	1.2	1.2
3	90	1.3	1.2
4	100	1.3	1.2
5	110	1.3	1.2
6	120	1.4	1.3
7	130	1.2	1.2
8	140	1.3	1.2
9	150	1.3	1.2
10	160	1.3	1.3
11	170	1.4	1.4
12	180	1.4	1.3
13	190	1.3	1.2
14	210	1.2	1.2
15	230	1.3	1.3
16	250	1.2	1.2
17	270	1.2	1.2
18	300	1.2	1.2
19	330	1.2	1.1
20	390	1.2	1.1
21	450	1.2	1.1

Figure 4.1 Flow Assessment by weight and volume

Table 4.6 Assessment of Flow by volume and weight (Exp 22: 6633-6)

Sample Number	Elapsed Time (minutes)	Flow Column A		Flow Column B	
		mL/min	Weight (grams)	mL/min	Weight (grams)
0	0	1.3	1.23	1.2	1.24
1	30	1.4	1.32	1.3	1.33
2	60	1.2	1.23	1.2	1.21
3	90	1.2	1.35	1.2	1.13
4	120	1.2	1.24	1.3	1.29
5	150	1.3	1.29	1.3	1.29
6	180	1.2	1.22	1.2	1.21
7	240	1.2	1.22	1.3	1.29
8	300	1.2	1.21	1.2	1.23
9	360	1.3	1.29	1.1	1.11
10	420	1.1	1.13	1.1	1.11
11	480	1.1	1.12	1.2	1.13
12	540	1.2	1.13	1.2	1.18

Bromide tracer breakthrough was first detected in the effluent at approximately 90 minutes at the target loading rate of 0.1m/hour (Table 4.5). Bromide breakthrough curves show some inconsistencies between experiments, but are consistent between columns at both ionic strengths (Figure 4.1 and 4.2). Columns were repacked with fresh media between experiments, and the variations in media characteristics (ES, D_{50}, UC) will affect porosity, possibly contributing to differences in breakthrough time (Table A4, Appendix A). A certain amount of heterogeneity in media was desirable to more closely mimic naturally occurring subsurface conditions. The duplicate columns were packed with media from the same batch for each trial. Slower flow was often observed in one column relative to the other within each experiment which would also alter breakthrough times.

The bromide tracer was passed through the column for approximately 160-180 minutes. At 0.1mM KCl, bromide tracer tests took approximately 6 hours as the falling limb was measured and graphed; for experiments conducted with a 10mM KCl AGW, the conductivity increased when the 10mM solution reached the effluent portal and bromide measurements ceased. The length of the bromide tracer process for the large diameter columns combined with flushing requirements resulted in bromide tracer

tests being conducted for only half the colloid trials. From the tracer tests conducted, the results generally agree with the theoretical calculation that one PV (114mL) would take approximately 96 minutes to pass through a column (Appendix B). To collect enough liquid sample for accurate measurement, approximately 2.5 minutes of flow was needed. In addition to changing sample collection tubes, the one-minute sample collection requirement for measurement of flow produced a minimum sample interval of eight or nine minutes using one laboratory personnel. In an effort to standardize sampling regimes, a 10 minute sample frequency was adopted. For future investigations, a two-person sampling team could more accurately pinpoint breakthrough by increasing the sample frequency to every 5 minutes, especially in the time range of expected breakthrough, which in this case would have required sampling at 85, 90, 95 and 100 minutes. A different probe, a flow-through cell or alternative measuring approach may further improve accuracy of tracer and colloid samples collected around breakthrough.

Table 4.7 Time to Breakthrough of Bromide Tracer

Trial #	Column A		Column B	
	Time to Breakthrough (minutes)	Flow Rate Range (mL/min)	Time to Breakthrough (minutes)	Flow Rate Range (mL/min)
1	105	1.2-1.4	100	1.1-1.2
2	105	1.2-1.4	110	1.2-1.3
3	90	1.7-1.9	90	1.5
4	100	1.4-1.8	90	1.4-1.7
5	90	1.4-1.7	100	1.4
6	90	1.4-1.6	90	1.4-1.8
8	90	1.3-1.4	90	1.3-1.4
10	90	1.5-1.8	90	1.4-1.6
12	90	1.3-1.5	90	1.3-1.4
14	90	1.4-1.6	90	1.3-1.4
22	90	n/a	90	n/a

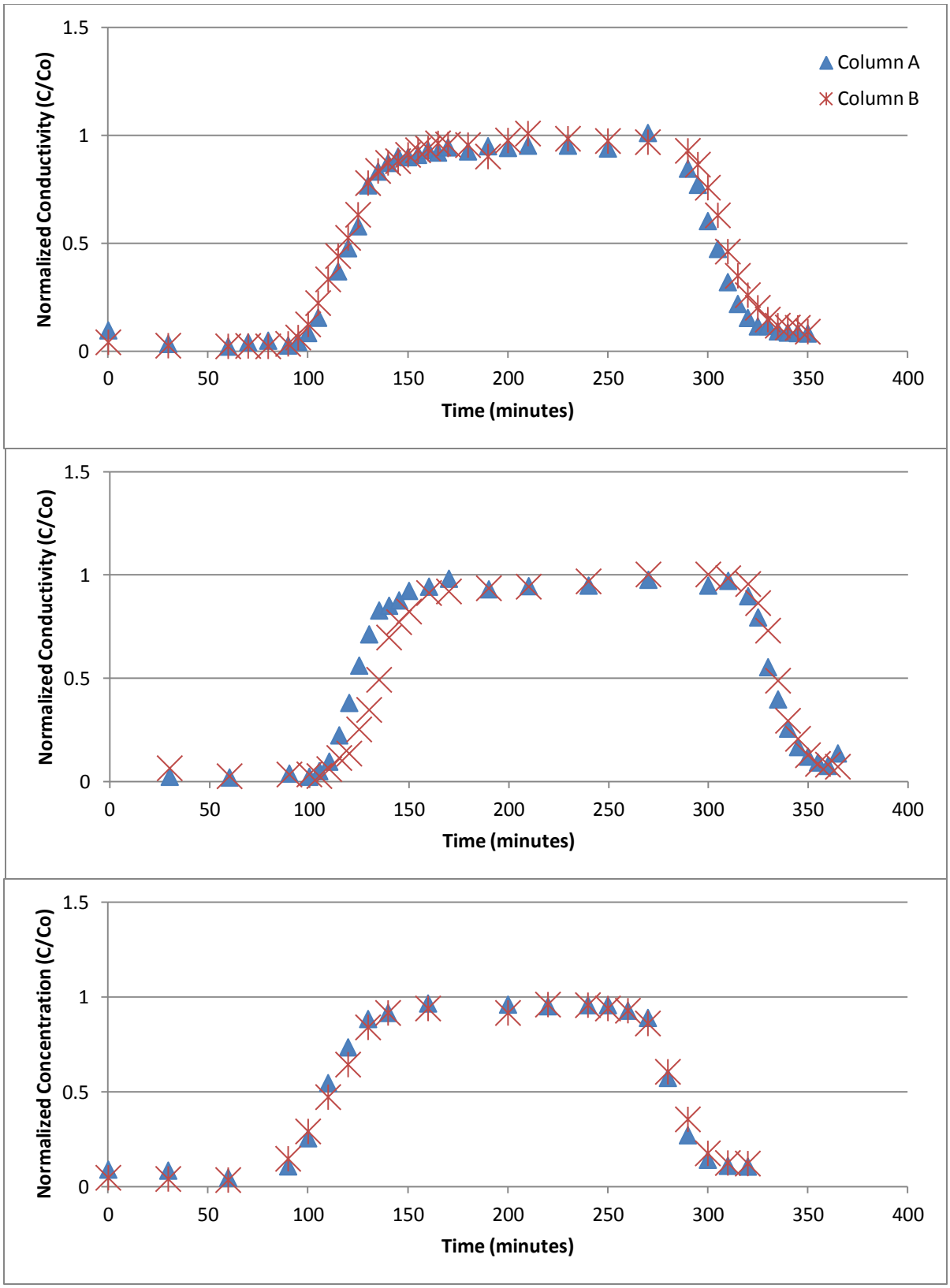

Figure 4.2 Bromide Breakthrough Curves at 0.10mM KCl

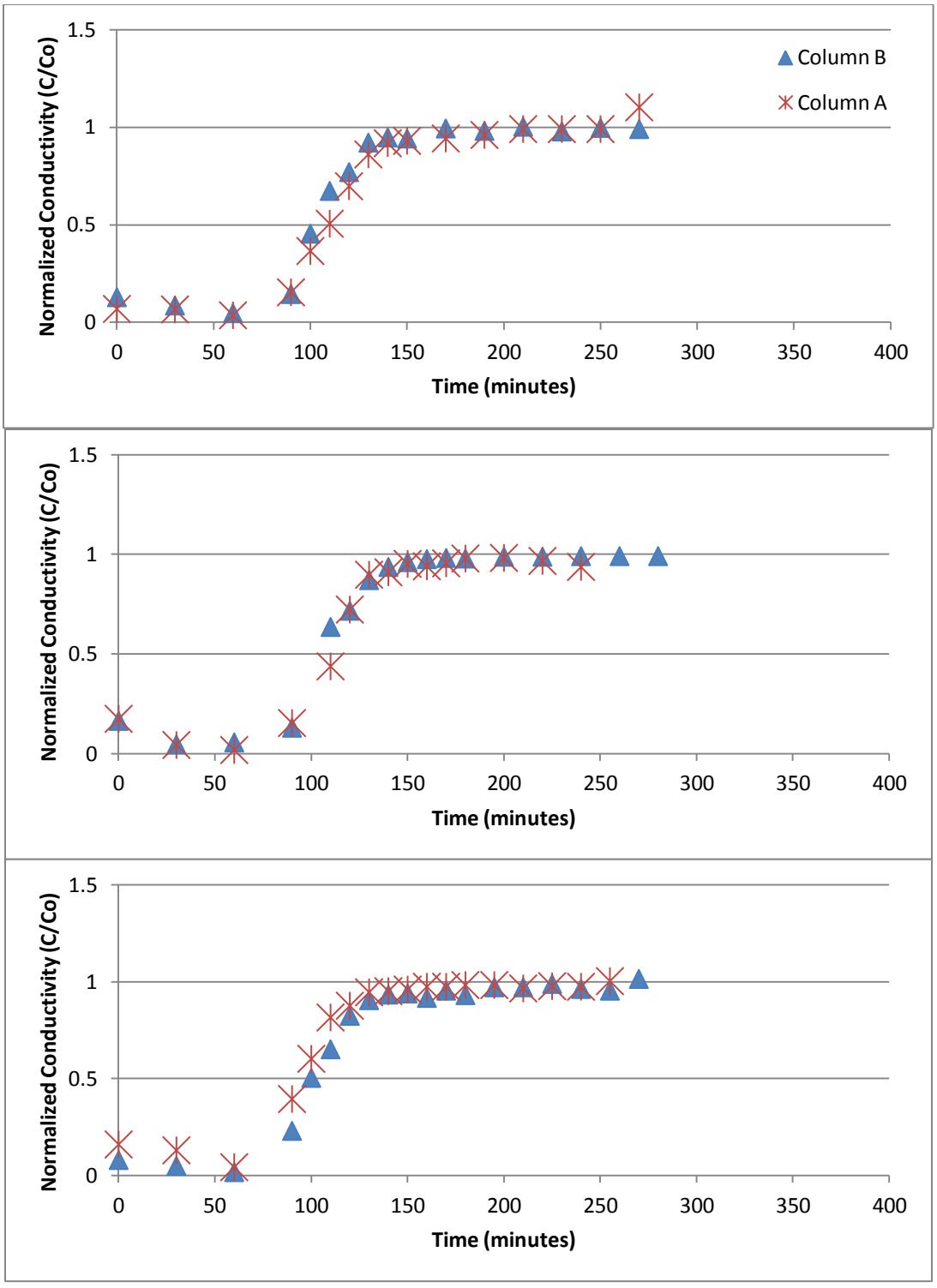

Figure 4.3 Bromide Breakthrough Curves at 10mM KCl

4.2.2 Dye Tracer Test

Brilliant blue FCF dye (4 grams/L; Colour Index 42090) was used to stain flow pathways for a visual assessment. The dye suspension was pumped into the columns using the same flow rate used in all experiments (1.2mL/minute). Both columns visually demonstrate plug flow-like behaviour until a media depth of approximately 7.5cm, when the uniform front becomes more ragged as mixing and dilution of the dye occurs (Figure 4.4: Column A). The dye arrived at the effluent portal prior to complete saturation of all column media with the dye, indicating that the small dye molecules have found preferential pathways of higher velocity. This partially explains the variability in the breakthrough time of the bromide tracer tests, and may indicate inconsistencies in media packing, or the stratification of separate media layers during column packing. A high variability in time-to-breakthrough may be due in part to large changes in the uniformity coefficient of media with each experiment.

Bacillus subtilis spores were observed to breakthrough earlier than the bromide tracer on several occasions; tracer ions such as dye and bromide may be able to diffuse into small pore spaces while (bio)colloids are forced into the bulk fluid traveling through larger pore spaces due to size exclusion, thereby resulting in earlier breakthrough of colloids (Sinton et al., 2000; Bradford et al., 2004). The fluctuating breakthrough times can be partially accounted for by the evidence of non-plug flow-like behaviour provided by this dye tracer and the large range in media characteristics used in these experiments.

Figure 4.4 Dye Tracer Test – Column A

4.3 Colloid Breakthrough

4.3.1 Unfavourable Conditions

The time to breakthrough (detection of (bio)colloids in filter effluent samples) of colloid suspensions in 0.10mM KCl was an average of 91 minutes for Column A and 93 minutes for Column B, in comparison with an average of 96 minutes for the Bromide tracer breakthrough (Table 4.6). Experiment 16 was an exception (YG 4.5 µm MS) in that MS were not present in the 90 minute sample but broke through in the next sample which was collected at 120. A truncated sampling regime was used (30 minute sampling frequency) for this experiment, so the exact time of colloid breakthrough remains undetermined but fell somewhere between 90 and 120 minutes.

For *Bacillus subtilis* spores, a pseudo steady-state (plateau) was achieved between 140 and 180 minutes with a time-to-plateau for Column A an average of 159 minutes, and 162 minutes for Column B. The earliest time-to-plateau was observed in the LT spore strains. Grab samples were used to determine breakthrough but sampling frequency was limited to 10 minute intervals due to methods used. The inconsistency in time to breakthrough and time to plateau may be due to variable flow rates (~1.1 to ~1.5mL/min), changing media characteristics, a combination of these two factors, or these factors in conjunction with (bio)colloid characteristics such as size and zeta potential. Kim et al. (2008a), suggested flow rate had the greatest effect on the

movement of B*acillus cereus* spores during laboratory experiments (as compared with grain size and pH). During RBF, subsurface flow rates may contribute to colloid residence time in aquifer materials but the scale is much larger (days) than during laboratory experimentation. Under the LT2ESWTR, to earn 0.5 log removal credit a well must be set back 7.5m from a surface water source (USEPA, 2006). According to Schijven et al., 2003, 0.5 log removal would require a minimum of 5 days travel under typical groundwater velocity (1.5m/day in dune sand) (Schijven et al., 2003) in comparison with the 1500mm of sand used in these experiments with 8 hour duration.

During two experiments at low ionic strength (Trial 2 and Trial 6), spore breakthrough was observed prior to breakthrough of the bromide tracer. This observation is in agreement with findings of Grolimund et al. (1998), who observed colloid breakthrough earlier than that of a nitrate tracer, which they attributed to the ability of the tracer to diffuse into small pore spaces that exclude the larger colloids (carboxyl latex particles). A higher frequency of sampling during expected breakthrough times would more clearly identify the relationship between colloid and tracer breakthroughs times. In many cases sampling for bromide tracers occurred at 30 minute intervals for the first 90 minutes, while sampling frequency for colloids decreased to 20 minute intervals after 60 minutes. A five minute sampling frequency between 70 and 100 minutes would have been more appropriate. If available, collecting samples using a fraction collector could also help isolate a precise time-to-breakthrough.

Table 4.8 Time to Colloid Breakthrough and Time to Effluent Pseudo Steady-State Plateau

				Column A			Column B		
				Time to Breakthrough (minutes)		Time to Plateau (min)	Time to Breakthrough (minutes)		Time to Plateau (min)
Exp #	Exp Code	Type	Sub-Spc	Bromide	Colloid	(min)	Bromide	Colloid	(min)
	0.10mM KCl								
1	MS1 NR	-	-	105	100	160	100	n/a	n/a
16	MS2 YG*	-	-	n/a	120	150	n/a	120	150
2	3610-1	WT	1	105	90	160	110	90	150
8	3610-2	WT	1	90	90	160	90	100	180
3	W23-1	LT	2	90	90	160	90	100	170
6	W23-4	LT	2	90	80	140	90	n/a	n/a
7	W23-5	LT	2	n/a	90	170	n/a	90	150
13	168-4	LT	1	n/a	70	150	n/a	70	150
21	168-1	LT	1	n/a	90	160	n/a	90	160
17	6633-2	WT	2	n/a	90	170	n/a	90	170
18	6633-3	WT	2	n/a	90	165	n/a	90	175

*sampling frequency = 30 minute intervals

4.3.1.1 Microspheres

Breakthrough curves show the filter effluent concentrations of microspheres (MS) as collected from the effluent portal. Using the percent removals from the last five samples collected, a mean $\log_{10}$ removal was determined (Table 4.7). The average removal of 1.1µm MS was 80% with a SD of 6.51% over the last five samples, or 0.71 $\log_{10}$ and ranged from 0.38 to 0.99 $\log_{10}$ over the course of the plateau (Figure 4.6), while the protozoan-sized MS (4.5µm) showed greater removal, with a mean of 3.4 and 3.5 $\log_{10}$ removal in Column A and B respectively (Figure 4.7).

Table 4.9 Percent and Mean Log_{10} Removal of 1.1µm and 4.5 µm Microspheres

Size		**1.1µm**	**4.5µm**	
Column		**A**	**A**	**B**
% Removal	1	80.51	99.97	99.97
	2	77.38	99.97	99.98
	3	89.79	99.96	99.97
	4	82.95	99.96	99.97
	5	72.28	99.96	99.96
Average (N=5)		80.58	99.96	99.97
SD		6.51	0.01	0.01
Mean Log Removal		0.71	3.40	3.50

These findings agree with theoretical predictions and experimental results; removal of 2 $\log_{10}$ to greater than 3.5 $\log_{10}$ is typically observed for colloids, and MS specifically, of approximately 4µm size (Passmore et al., 2010; Schijven et al., 2003; Emelko, 2001). Greater removal of larger colloids is consistent with CFT in that colloids near the minimum contact efficiency (~1µm in size) will be transported to collector surfaces to a lesser extent in saturated porous media (Ryan and Elimelech, 1996). Breakthrough of the smaller MS occurred at 100 minutes. Breakthrough of the larger MS was observed at 120minutes. Both 1.1µm and 1.5µm MS reached a pseudo-steady state plateau at 140 and 150 minutes, respectively. Experiment 16 (YG 4.5µm MS) was half the length of Experiment 1 (NR 1.1 µm MS), which was used to design the experimental sampling schedule.

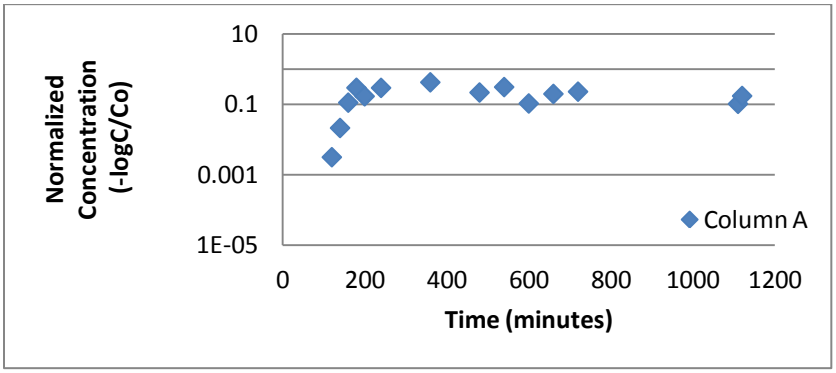

Figure 4.5 Breakthrough of Nile Red 1.1µm Microspheres in 0.10mM KCl

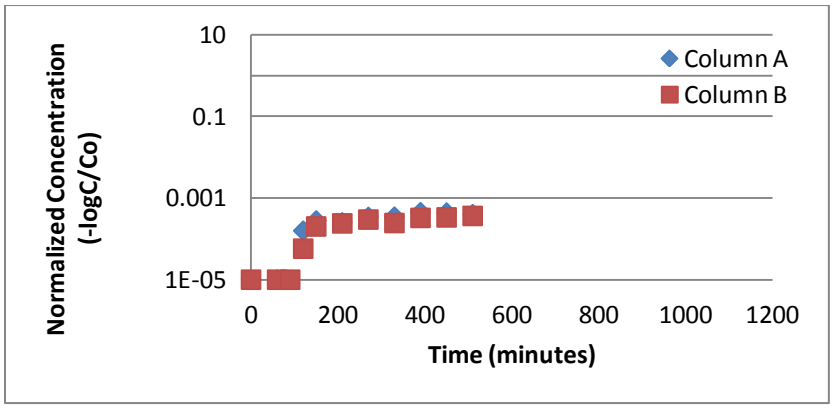

Figure 4.6 Breakthrough of YG 4.5µm Microspheres in 0.10mM KCl

4.3.1.2 3610: Wild-Type Bacillus subtilis *subspecies* subtilis

Experiment three assessed the transport of WT spores of *Bacillus subtilis* subspecies *subtilis*. Two trials were performed (3610-1 and 3610-2) in 0.10mM KCl with time-to-breakthrough occurring at 90 minutes in Trial 1, and 90 and 100 minutes in Trial 2 for Column A and B, respectively. A sampling interval of 10 minutes did not allow further clarification of the time-to-breakthrough. The time required to reach a pseudo steady-state plateau was 160 minutes for both trials in Column A, and 150 and 180 minutes in Column B in Trial 1 and 2 respectively (Table 4.6; Figure 4.7). Some possible reasons for these differences include varying media characteristics including D_{50} and UC, and differences in the column equipment. Flow in Column B was generally 0.2 to 0.4mL per minute slower than in Column A, and tended to fluctuate. Although the column tubing was the same length, it is possible that sealing them with zip ties resulted in pressure at tubing junctions. The Columns were also sealed using rubber O-rings that wore down gradually and were uniform but not identical in sizing.

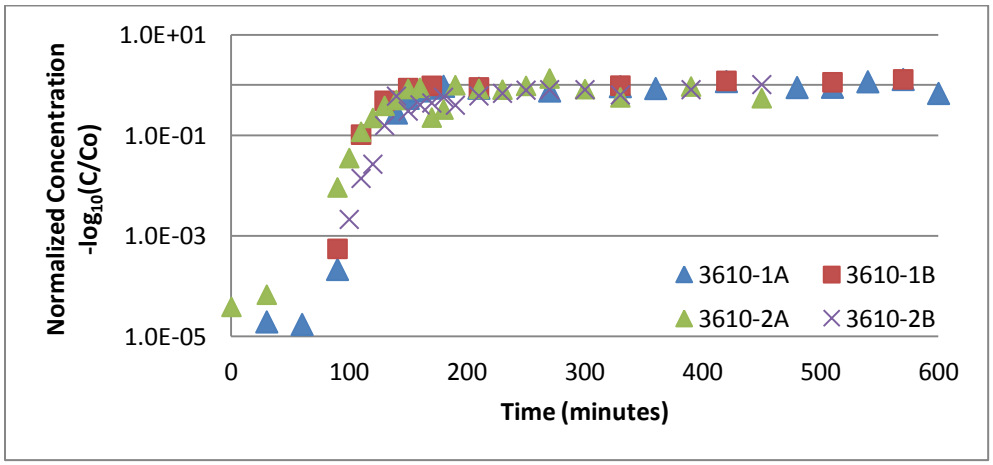

Figure 4.7 *Bacillus subtilis subtilis* WT Log Removal in 0.10mM KCl

Mean $\log_{10}$ removal of WT 3610 under unfavourable conditions was negligible (Table 4.10; Figure 4.8) as expected for colloids in this size range. Good reproducibility was observed both between Trials and Columns although several negative values were calculated for percent removal which may indicate issues with feedstock calculations (Figure 4.8). Several outliers were observed which may be attributable to the spread-plate methods used to calculate spore concentrations.

Table 4.10 Percent and Mean Log_{10} Removal of WT 3610 in 0.10mM KCl

Trial	1		2	
Column	A	B	A	B

% Removal	1	5.79	9.08	-36.76	17.94
	2	13.46	1.41	15.99	17.94
	3	-19.04	-22.69	42.36	35.53
	4	9.44	-15.02	5.24	17.94
	5	8.35	11.82	44.51	-3.55
Average (N=5)		3.60	-3.08	14.27	17.16
SD		12.95	15.14	33.14	13.86
Mean Log Removal		0.02	-0.01	0.07	0.08

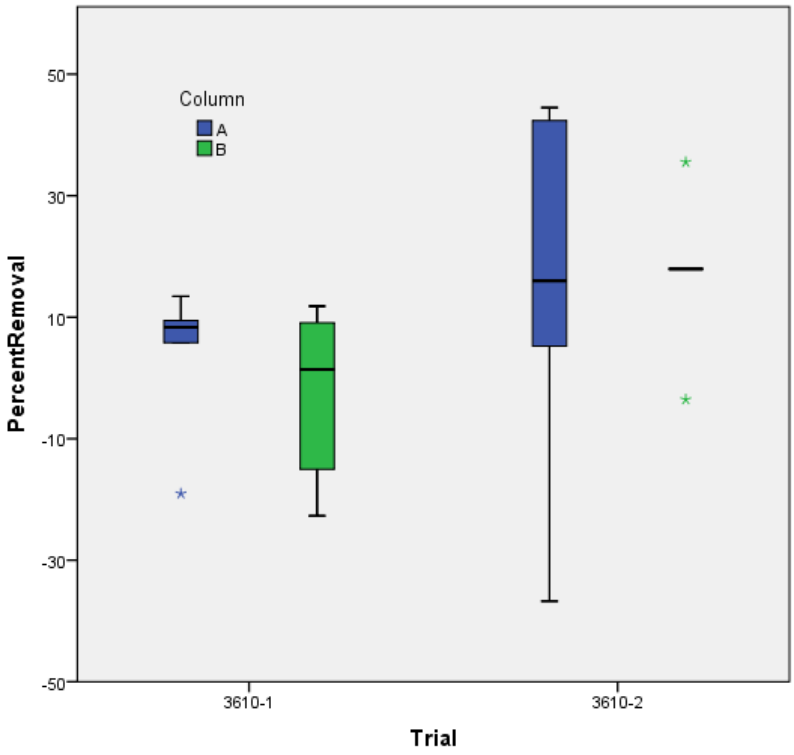

Figure 4.8 Percent Removal of WT 3610 in 0.10mM KCl

4.3.1.3 *W23: Laboratory-Type* Bacillus subtilis *subspecies* spizizenii

Experiment four examined the retention of LT spores of *Bacillus subtilis* subspecies *spizizenii* in 0.10mM KCl during three trials (W23-1, W23-4, W23-5). The time to breakthrough observed with this strain was ~80 to 100 minutes with three of the five replicate trials reaching breakthrough at ~90 minutes (Table 4.11; Figure 4.9). The time to reach a pseudo steady-state plateau was 170 minutes for four of five trails. The calculated stock concentrations were consistent, measuring 365,385, 379, 856, and 352,589 CFU/mL for Trials 1, 4 and 5 respectively, however some normalized

concentration calculations (C/C_0) generated values greater than 1, indicating a potential problem with stock concentration calculations, sample handling or methodology.

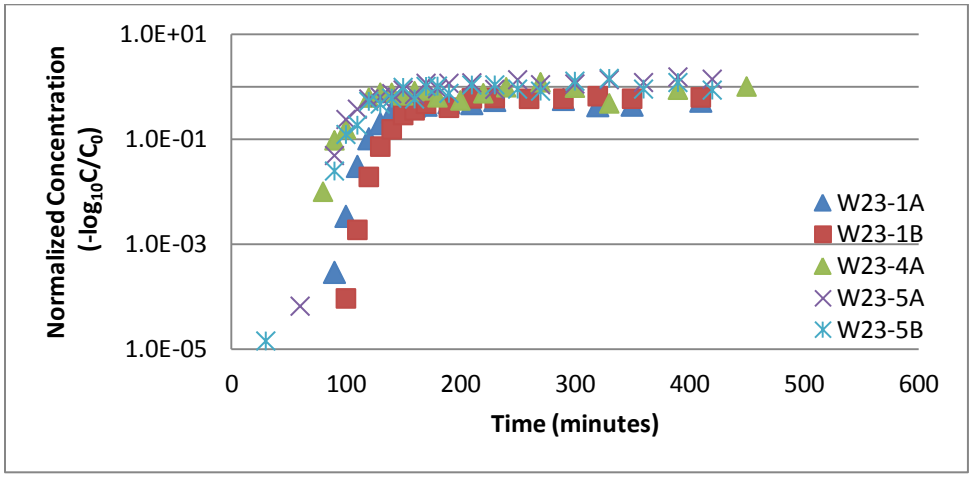

Figure 4.9 *Bacillus subtilis spizizenii* LT W23 Log Removal in 0.10mM KCl

Average percent removals ranged between 39 and 49% for Trial 1 (Table 4.11; Figure 4.10), which showed the lowest SD in calculated spore concentrations, and mean $\log_{10}$ removals of 0.22 and 0.30 for Column A and B respectively. Trials 4 and 5 show little to no mean $\log_{10}$ removal which is expected for colloids in this size range in unfavourable deposition conditions, but have widely ranging concentrations and large SDs. Trials 4 and 5 were conducted 7 months after Trial 1, potentially suggesting that spores responded to their storage conditions in some way. If spores initiated or increased EPS production in an effort to increase the likelihood of transport to more favourable growth conditions, steric repulsion between media and spores may have increased, reducing spore retention in the latter trials. Measuring spore electrophoretic mobility before and after storage and prior to each experiment may identify changing spore properties and assist in explaining the variations in spore retention observed in this investigation.

Table 4.11 Percent and Mean Log_{10} Removal of LT W23

[KCl]		0.10mM					
Trial		1		4		5	
Column		A	B	A	B	A	B
% Removal							
	1	45.81	41.89	-19.78	n/a	-11.46	-25.74
	2	43.44	41.61	5.23	n/a	-34.43	-42.28
	3	57.03	34.82	52.50	n/a	-18.55	11.13
	4	56.03	41.71	13.30	n/a	-51.45	-18.65
	5	47.64	38.24	0.40	n/a	-37.27	14.92
Average (N=5)		49.99	39.65	10.33	n/a	-30.63	-12.12
SD		6.16	3.10	26.55	n/a	15.85	24.54
Mean Log Removal		0.30	0.22	0.05	n/a	-0.12	-0.05

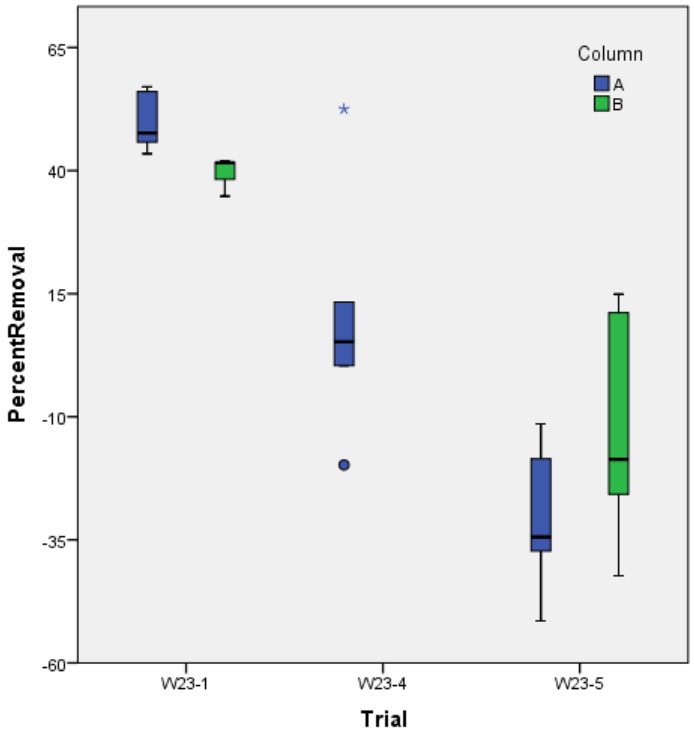

Figure 4.10 Percent Removal of LT W23 in 0.10mM KCl

4.3.1.4 *168: Laboratory-Type* Bacillus subtilis *subspecies* subtilis

Two trials were conducted with LT spores of *Bacillus subtilis* subspecies *subtilis* in 0.10mM KCl (168-1, 168-4). The time to breakthrough observed in Experiment Five was 70 minutes for both Columns in trial 168-4 and 90 minutes for 168-1 with time to

plateau at 150 and 160 minutes for trial 4 and 1 respectively (Table 4.12; Figure 4.12). Complete breakthrough (no removal) was observed in both trials for LT 168.

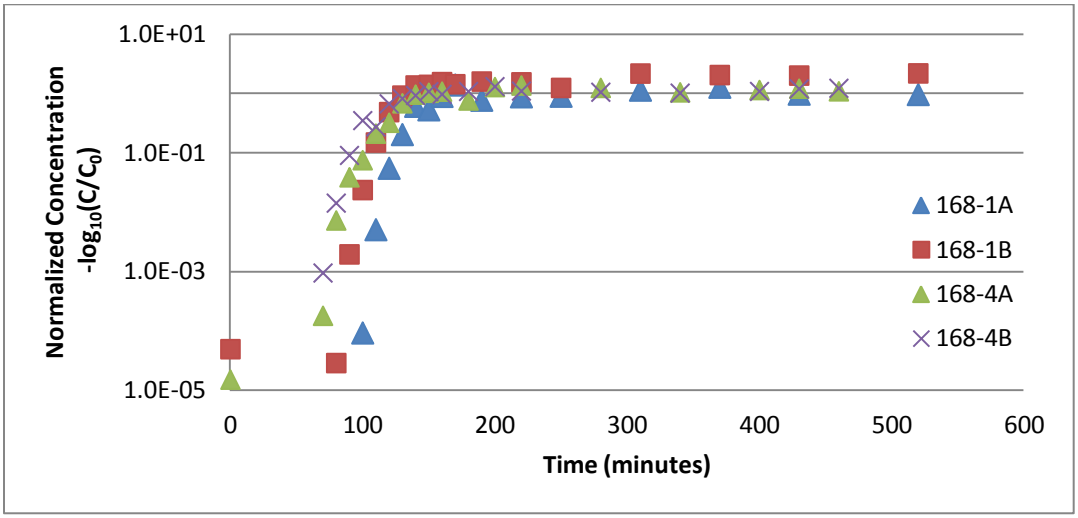

Figure 4.11*Bacillus subtilis* subtilis LT 168 Log_{10} Removal in 0.10mM KCl

Table 4.12 Percent and Mean Log_{10} removal of *Bacillus subtilis subtilis* 168

[KCl]		0.10mM			
Trial		1		4	
Column		A	B	A	B
% Removal	1	-12.72	-22.84	-3.93	-22.99
	2	-26.22	-114.63	-0.56	-3.55
	3	3.48	-102.48	-7.29	-13.27
	4	4.83	-98.43	-18.5	-19.25
	5	-61.99	-115.98	-21.12	-7.85
Average (N=5)		-18.52	-90.87	-10.28	-13.38
SD		27.44	38.78	9.06	7.13
Mean Log Removal		-0.07	-0.28	-0.04	-0.05

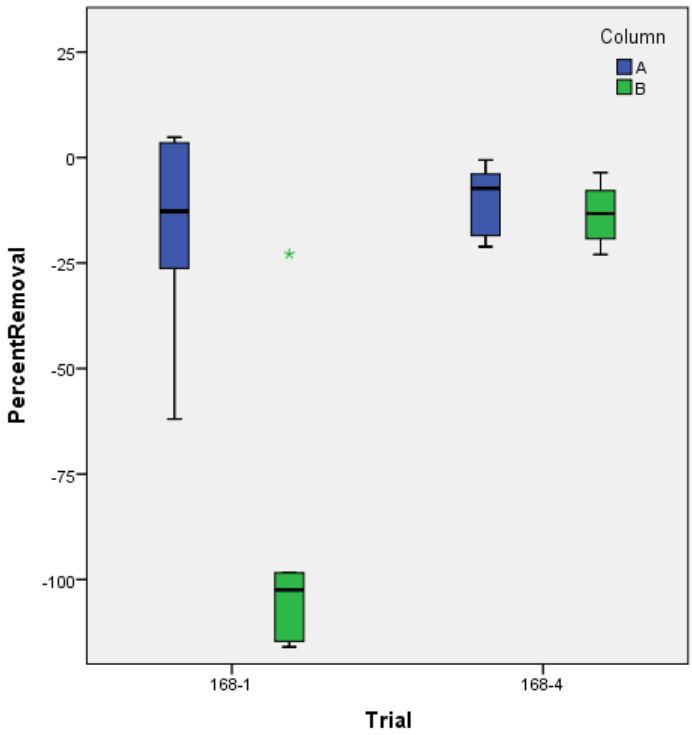

Figure 4.12 Percent Removal of LT *Bacillus subtilis subtilis* 168 in 0.10mM KCl*
*note change in scale due to suspected stock concentration miscalculation

Trial 1 with LT 168 and particularly Column B shows the importance of accurate stock concentration counts, as normalized concentrations above 1 are theoretically impossible; sample handling errors may account for part of this along with inadequate mixing of the stock solution prior to sample collection during this trial. There is good reproducibility between the two trials conducted in Column A, however the exteme values calculated for Column B and high SD observed for trial 1 suggest human error contributed to a range of calculated removals using the serial dilution and plate count method for concentration estimates. Full breakthrough was expected and observed therefore these trials were not repeated.

4.3.1.5 *6633: Wild-Type* Bacillus subtilis *subspecies* spizizenii

Experiment Six examined the retention of WT spores of *Bacillus subtilis* subspecies *spizizenii* 6633 in 0.10mM KCl during two trials (6633-2, 6633-3). Breakthrough was consistently observed at 90 minutes, with time-to-plateau at 90 minutes (Table 4.6, Figure 4.13). The time to reach a pseudo steady-state plateau was 170 minutes for both Columns in Trial 6633-2, and 165 and 175 minutes for Column A and B during Trial 6633-3 respectively. Although sampling frequency was a 10 minute interval, due to an error in sample collection, samples were collected at 155, 165, and 175 minutes instead of 160 and 170 minutes making it difficult to identify an exact time-to-breakthrough. Changing media characteristics may have also contributed to differences in breakthrough times.

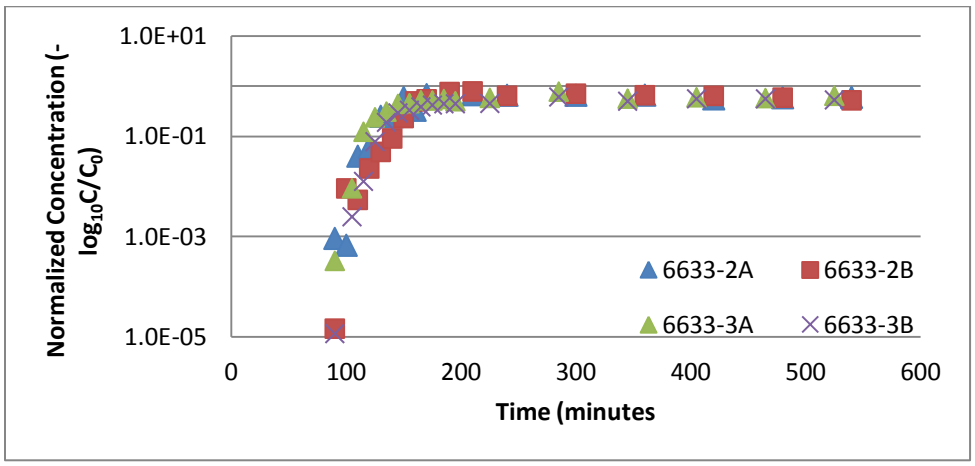

Figure 4.13 *Bacillus subtilis spizizenii* WT 6633 Log Removal in 0.10mM KCl

There was very good reproducibility between the both the columns and trials with means of ~0.20 $\log_{10}$ removal for all four trials, and low SD values (Table 4.13; Figure 4.14). This strain showed the highest removal of spores under the low ionic strength conditions and the most consistent results. All trials with WT 6633 were conducted in rapid succession (within a one-month time frame) and these two trials were conducted within 4 days of each other. This suggests that spore characteristics (not measured here) may vary as spores age and adapt to storage conditions. If personnel are available, conducting Trial repeats within a short time (days and weeks) may increase reproducibility of results and more accurately estimate removals. Repeating Trials over a longer time period (months and years) may demonstrate the range of transport that might be observed within a spore strain and type. The consistencies displayed by this WT strain, and the rapid succession of trials hints that spore age or ability to

adapt to storage conditions may play an integral role in transport behaviour and merits further investigation.

Table 4.13 Percent and Mean Log_{10} Removal of WT 6633

[KCl]		0.10mM			
Trial		2		3	
Column		A	B	A	B
% Removal					
	1	34.44	29.57	22.50	39.46
	2	34.11	34.44	43.81	50.23
	3	44.50	36.22	40.18	44.30
	4	41.58	41.25	42.36	44.42
	5	40.28	48.07	35.94	47.08
Average (N=5)		38.98	37.91	36.96	45.10
SD		4.56	7.05	8.61	3.98
Mean Log Removal		0.21	0.21	0.20	0.26

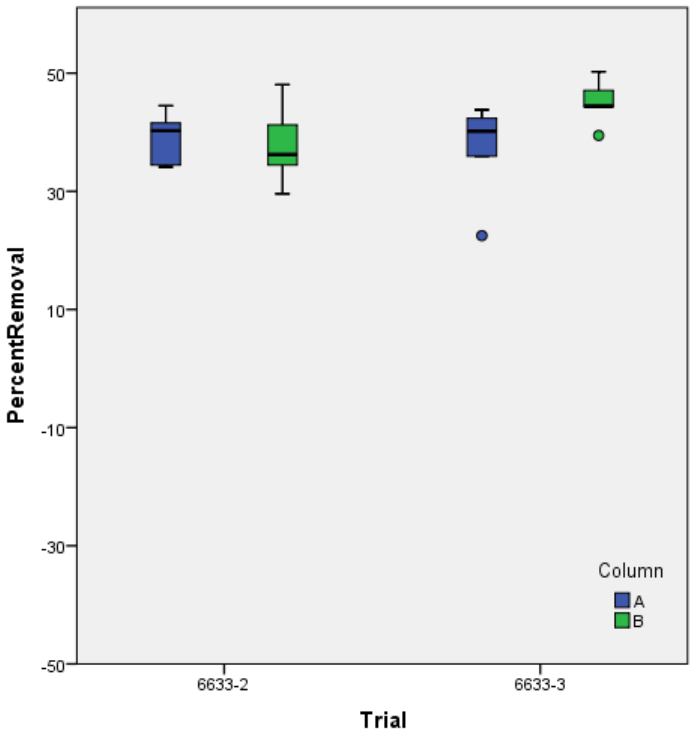

Figure 4.14 Percent Removal of WT 6633 in 0.10mM KCl

4.3.1.6 Conclusions

In 0.10mM KCl, or unfavourable conditions for deposition, little removal of the various spore strains was observed. When grouped, greater removal was seen from the two strains (6633 and W23) from subspecies *spizizenii* (Figure 4.15; Table 4.14) which showed 0.12 $\log_{10}$ removal in comparison with no removal from the spores from subspecies *subtilis*. The same pattern was observed when comparing WT spores with LT spores; spores of LT 168 from *Bacillus subtilis* subspecies *subtilis* showed complete breakthrough (no removal) while WT 3610 of the same subspecies showed a mean of 0.04 $\log_{10}$ removal. This suggests that LT spores from the more domesticated strains of *Bacillus* would provide the most conservative estimates of (bio)colloid retention in saturated porous media. However, all spore removals by filtration in unfavourable deposition conditions (0.10mM KCl) were lower than the removals of 1.1 and 4.5 µm microspheres in the same AGW (mean removals of 0.71 and ~3.5 log respectively) which suggests that MS or a (bio)colloid other than the spores of *Bacillus subtilis* may be a more appropriate surrogate for *Cryptosporidium parvum* transport through saturated porous media. As expected, the 4.5µm MS were removed to the greatest extent during these experiments and as (bio)colloids closest in size to the oocysts of *C. parvum,* suggesting that size may be the most important factor in removal. This investigation did not involve the quantification of *C. parvum* oocysts through the selected media and further investigation is warranted to determine if other (bio)colloids can more closely mimic oocyst transport.

Table 4.14 (Bio)Colloid Mean Log_{10} Removal

0.10mM KCl

Colloid	Mean	N	SD
NR MS 1.1	.71	5	.03
YG MS 4.5	3.48	10	.00
WT 3610	.04	20	.10
LT W23	.05	25	.19
LT 168	.00	20	.22
WT 6633	.22	20	.03

***Bacillus subtilis* LogRemoval**

Strain Type			
WT	.12	40	.11
LT	.00	45	.25
SubSpecies			
Subtilis	.00	40	.21
Spizizenii	.12	35	.15

*N = number of observations

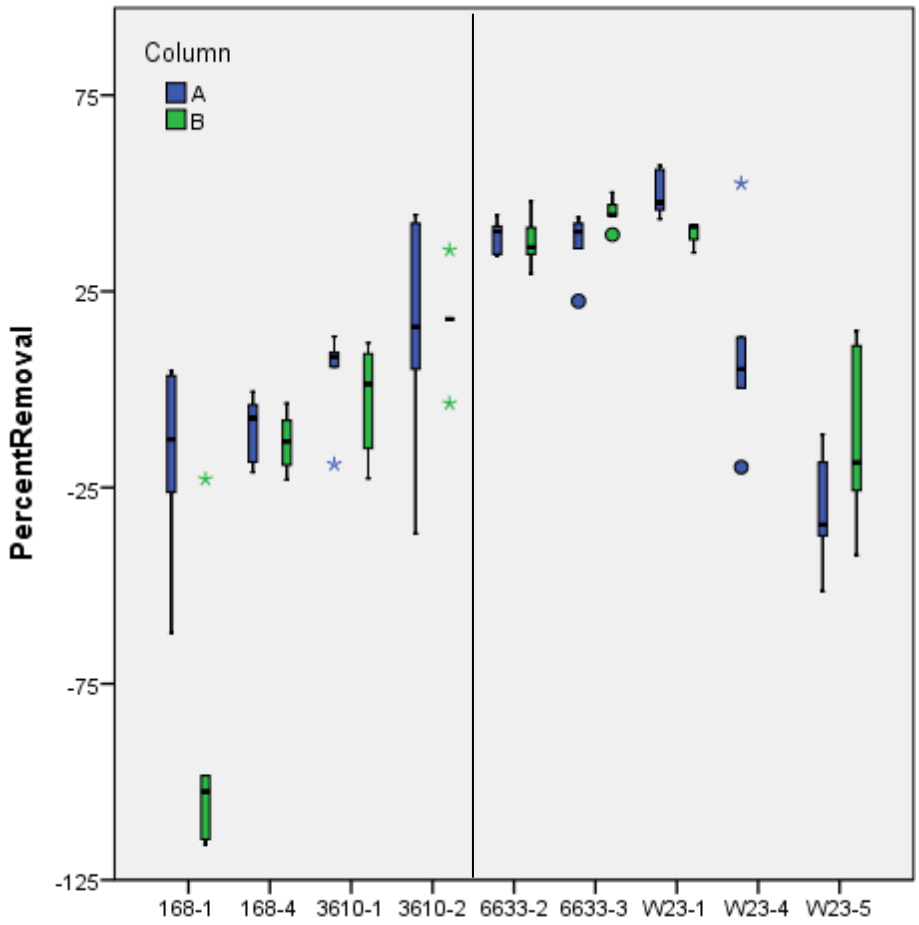

Figure 4.15*Bacillus subtilis* spore removal for all experiments conducted in 0.10mM KCl (left = *subtilis*, right = *spizizenii)*

4.3.2 Favourable Conditions

In 10mM KCl, the observed time to spore breakthrough ranged between ~80 and ~100 minutes depending on Column flow rates, media characteristics and sampling schedule employed. The time to reach a pseudo steady-state plateau was generally assumed to be between 120 and 180 minutes, however, data from Experiment 6 (WT 6633) suggest that the duration of the experiments precluded the evaluation of pseudo steady-state spore removal because sampling finished before a plateau was reached. Data collected here may outline a slowly rising limb relative to the results obtained in unfavourable spore-deposition conditions. Mean $\log_{10}$ removal results for all strains were higher than at unfavourable conditions, as expected.

4.3.2.1 *3610: Wild-Type* Bacillus subtilis *subspecies* subtilis

The transport of WT spores of *Bacillus subtilis* subspecies *subtilis* in 10mM KCl was examined during two trials (3610-3 and 3610-4). The time-to-breakthrough occurred at 80 and 90, and 70 and 80 minutes for Column A and B during Trial 3 and 4 respectively (Figure 4.16). Slower flow in Column B, and a ten minute sampling regime account for the discrepancy in breakthrough times. The time to reach a pseudo steady-state plateau was 140 minutes for Column A and B during Trial 3610-3. Column A reached a pseudo steady-state plateau before Column B, at 100 minutes, during Trial 3610-4, with Column B reaching its plateau at 130 minutes. A likely cause of this is higher flow in Column A (>1.4mL/min) in comparison with ~1.2mL/ minute in Column B, which is consistent with findings from Kim et al. (2008), who found spore transport increased by 71% when flow rate increased from 1.3mL/min to 3.0mL/min.

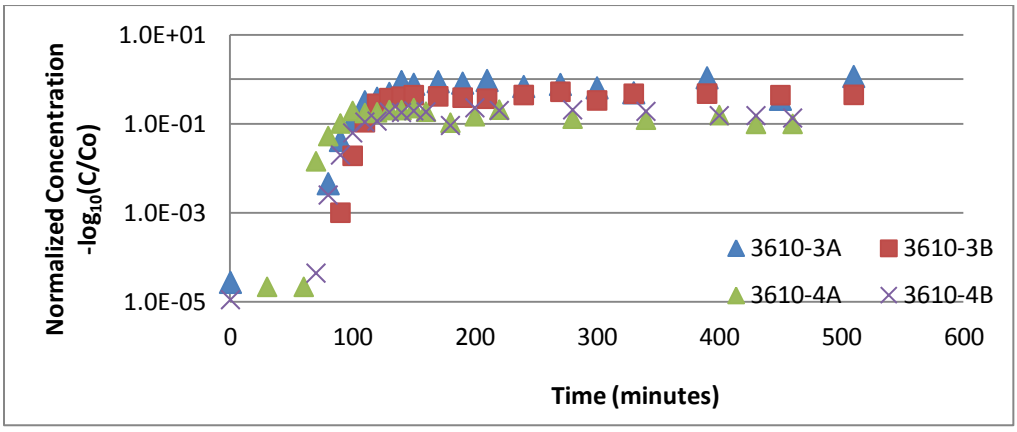

Figure 4.16 Removal of *Bacillus subtilis subtilis* WT in 10mM KCl (log_{10})

Mean log_{10} removals neared 1 log_{10} during Trial 2, with 0.92 and 0.78 log_{10} for Column A and B respectively with a low SD for both Columns and a very consistent plateau (Table 4.15). In contrast, Column A showed a very high SD of 35.28 with large fluctuations in the calculated concentrations over the last 3.5 hours. Prior to the sample collection at 330 minutes, concentrations were very stable with percent removal averaging 20%. Data such as these suggest a longer sampling period was required. It also may indicate issues with consistency of the feed stock concentrations, improper mixing, settling, or variable intake through the peristaltic tubing. During Trial 4, percent removals are consistent after 130minutes only increasing from 81% to 86% but the Trial ran for 460 minutes in comparison with 510 for Trial 3. The data from Trial 4 gives the impression of a plateau but could also indicate a slowly rising

limb, suggesting further experimentation with longer sampling schedules and larger stock volumes is required.

Table 4.15 Percent and Mean Log_{10} Removal of WT 3610 in 10mM KCl

Trial		3		4	
Column		A	B	A	B
% Removal	1	36.15	66.70	87.02	79.54
	2	50.60	52.98	87.68	81.08
	3	-7.39	52.62	84.38	84.82
	4	64.69	56.28	90.10	84.82
	5	-14.71	55.54	90.10	86.58
Average (N=5)		25.87	56.82	87.85	83.36
SD		35.28	5.74	2.39	2.93
Mean Log Removal		0.13	0.36	0.92	0.78

Mean log_{10} removals ranged between 0.13 and 0.92 but outliers were observed in two of four trials (Figure 4.17).

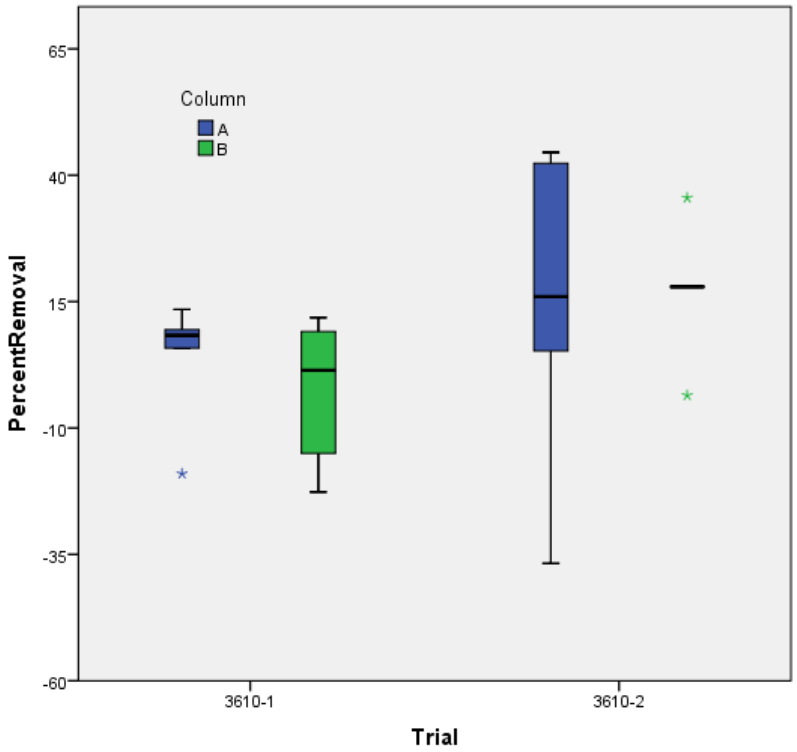

Figure 4.17 Percent Removal of WT 3610 in 10mM KCl

4.3.2.2 *W23: Laboratory-Type* Bacillus subtilis *subspecies* spizizenii

Experiment four examined the retention of LT spores of *Bacillus subtilis* subspecies *spizizenii* in saturated porous media. Two trials were conducted in 10mM KCl (W23-2, W23-3). The time to breakthrough observed with this strain was 90 minutes and the time to reach a pseudo steady-state plateau was 140 minutes during Trial 2, and 160 during Trial 3 (Figure 4.18), with differences possibly caused by media changes and flow fluctuations. Feed suspension spore concentration may have contributed to large difference in removal during this experiment; Trial W23-2 had a feed concentration of ~430878 CFU/mL in comparison to 1039445 CFU/mL during Trial W23-3; attachment sites available on the media may not have been saturated by the spore stock concentration used in Trial 2, allowing a higher percentage of total spores introduced into the columns to be retained within the media (Table 4.16; Figure 4.19).

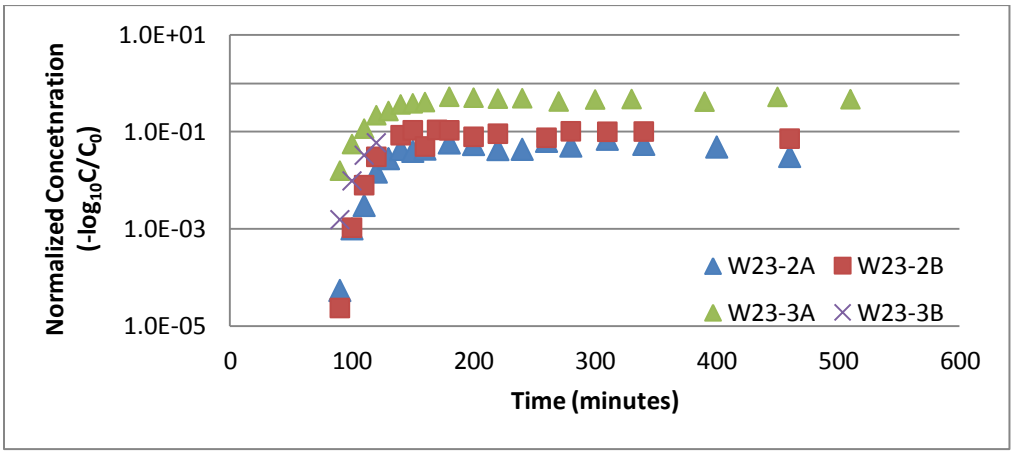

Figure 4.18 Removal of *Bacillus subtilis spizizenii* LT in 10mM KCl (log_{10})

Table 4.16 Percent and Mean $\log_{10}$ Removal of LT W23

[KCl]		10mM			
Trial		2		3	
Column		A	B	A	B
% Removal					
	1	94.93	92.50	53.82	n/a
	2	93.00	89.79	52.86	n/a
	3	94.51	90.02	58.31	n/a
	4	95.16	89.90	47.73	n/a
	5	96.91	92.81	53.18	n/a
Average (N=5)		94.90	91.00	53.18	n/a
SD		1.40	1.51	3.76	n/a
Mean Log Removal		1.29	1.05	0.33	n/a

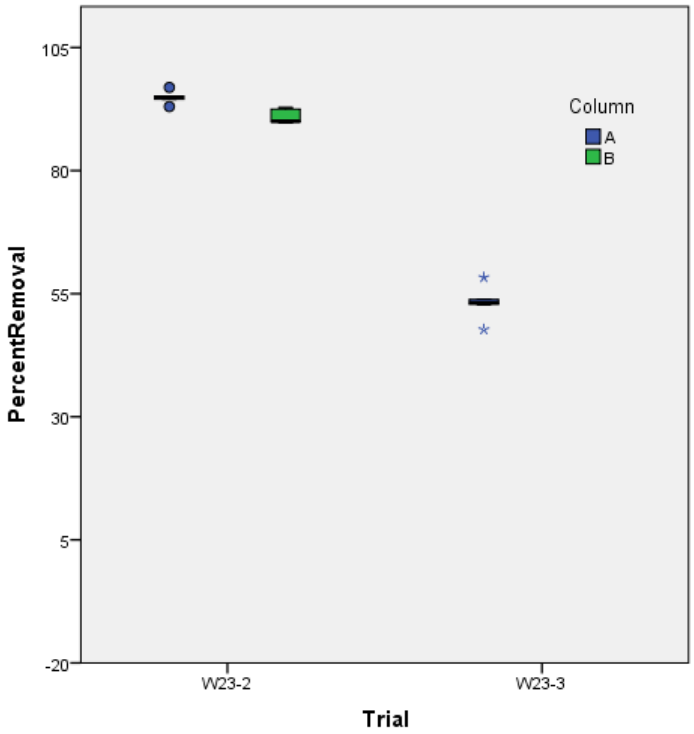

Figure 4.19 Percent Removal of LT W23 in 10mM KCl

4.3.2.3 *168: Laboratory-Type* Bacillus subtilis *subspecies* subtilis

Experiment Five investigated the retention of LT spores of *Bacillus subtilis* subspecies *subtilis* in saturated porous media under favourable deposition conditions (10mM KCl) (168-2, 168-3 and 168-5). The time to breakthrough observed in Experiment Five was 80, 90, and 100 minutes for Trial 2, 3 and 5 respectively (Figure 4.20). The overall

mean $\log_{10}$ removal of LT 168 was ~1 $\log_{10}$ in Trial 2, when a very low stock concentration was used. Trial 168-2, had a feed suspension concentration of 3857 CFU/mL due to a miscalculation, in comparison with Trials 163-3 and 168-5 which had concentrations closer to the targeted concentration ($1.0x10^6$ CFU/mL) of 1,050,500 and 651,167 CFU/mL respectively. This suggests incomplete saturated of the media for that trial and complete saturation of all attachment sites for Trial 3 and 5. Trials 3 and 5 show good agreement between the Columns A and B within the trial, but poor but acceptable reproducibility between the trials, with a 25% difference between the average percent removals (Table 4.17; Figure 4.21). Trials 3 and 5 also show high SDs, which may suggest sample handing problems, a false plateau or demonstrate the impact of varying media characteristics.

Table 4.17 Mean Percent and Log_{10} Removal of LT 168 spores in 10mM KCl

[KCl]		10mM					
Trial		2		3		5	
Column		A	B	A	B	A	B
% Removal	1	87.21	92.83	39.55	62.78	75.17	80.80
	2	91.27	93.47	12.58	22.42	61.10	60.33
	3	86.78	93.21	7.98	7.03	55.72	48.55
	4	92.01	95.42	39.87	2.11	32.17	31.66
	5	92.35	96.03	56.37	31.14	51.43	38.09
Average (N=5)		89.92	94.19	31.27	25.10	55.12	51.89
SD		2.71	1.43	20.40	24.08	15.64	19.48
Mean Log Removal		1.00	1.24	0.16	0.13	0.35	0.32

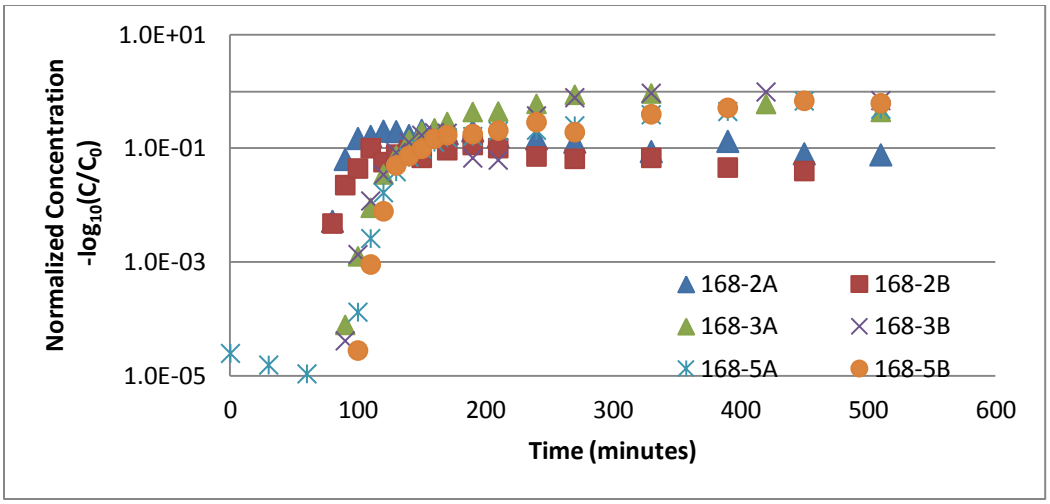

Figure 4.20 Removal of *Bacillus subtilis* LT 168 in 10mM KCl (log_{10})

Although Trial 2 did show nearly 1 log_{10} removal, the low stock concentration may have provided an unrealistic estimataion of removal. Trial 3 and 5 may be more representative of removals that could be expected at higher spore concentrations (Figure 4.21).

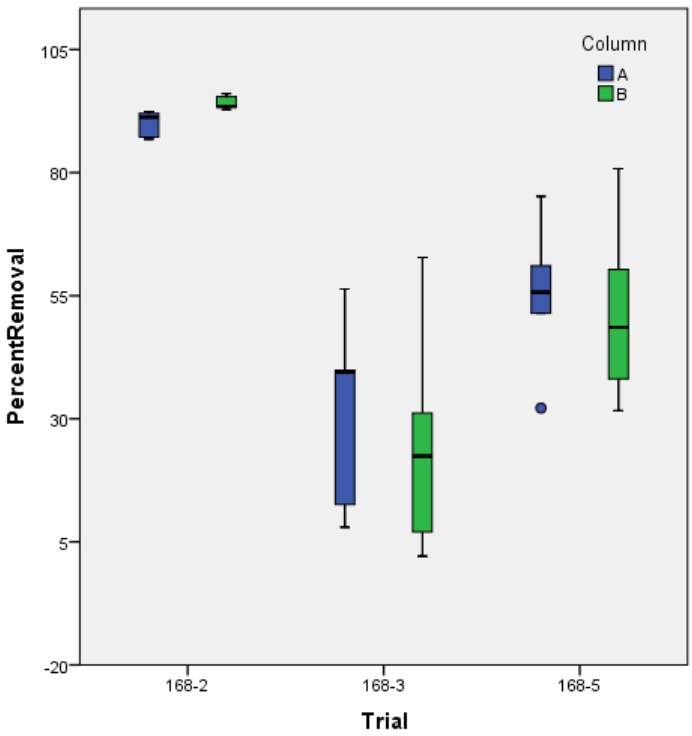

Figure 4.21 Percent Removal of LT 168 in 10mM KCl

4.3.2.4 *6633: Wild-Type* Bacillus subtilis *subspecies* spizizenii

Experiment Six examined the retention of WT spores of *Bacillus subtilis* subspecies *spizizenii* 6633 in 10mm KCl during four trials (6633-1, 6633-4, 6633-5, 6633-6). Breakthrough was consistently observed at ~80 minutes for Trial 1, 4 and 5 with breakthrough confirmed at 90 minutes for Trial 6, when no sample was collected at 80 minutes (Trial 6633-6 employed a reduced sampling frequency with a 30 min sampling interval).

Trial 1 appeared to reach a plateau, with filter effluent samples collected between 100 and 200 minutes resulting in a reasonable calculation of $\log_{10}$ removal ranging between 0.13 and 0.22 for the duration of this sampling period (Figure 4.22a). However, filter effluent spore concentrations increased steadily between 220 and 460 minutes for both columns. Trial 4 shows a similar trend (Figure 4.22b) with a potential plateau between 100 and 200 minutes, whereas trials 5 and 6 more clearly show a slowly rising limb, reaching a 'secondary' or true plateau nearer 4 hours (Figure 4.23).

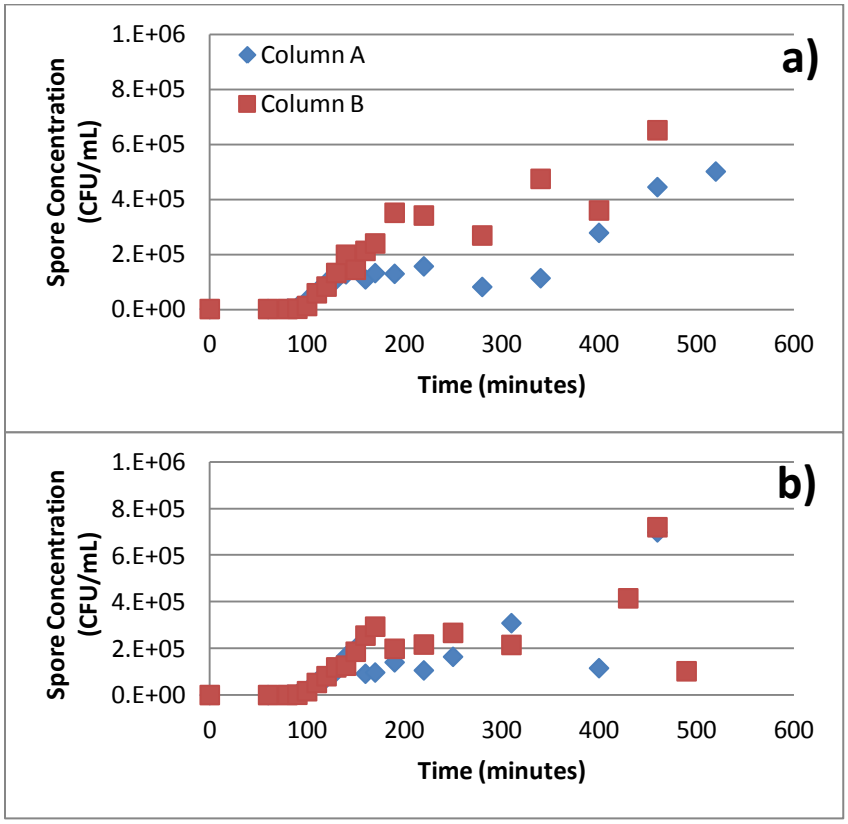

Figure 4.22 Effluent Spore Concentrations of WT 6633 during Trial 1 (a) and 4 (b) in 10mM KCl

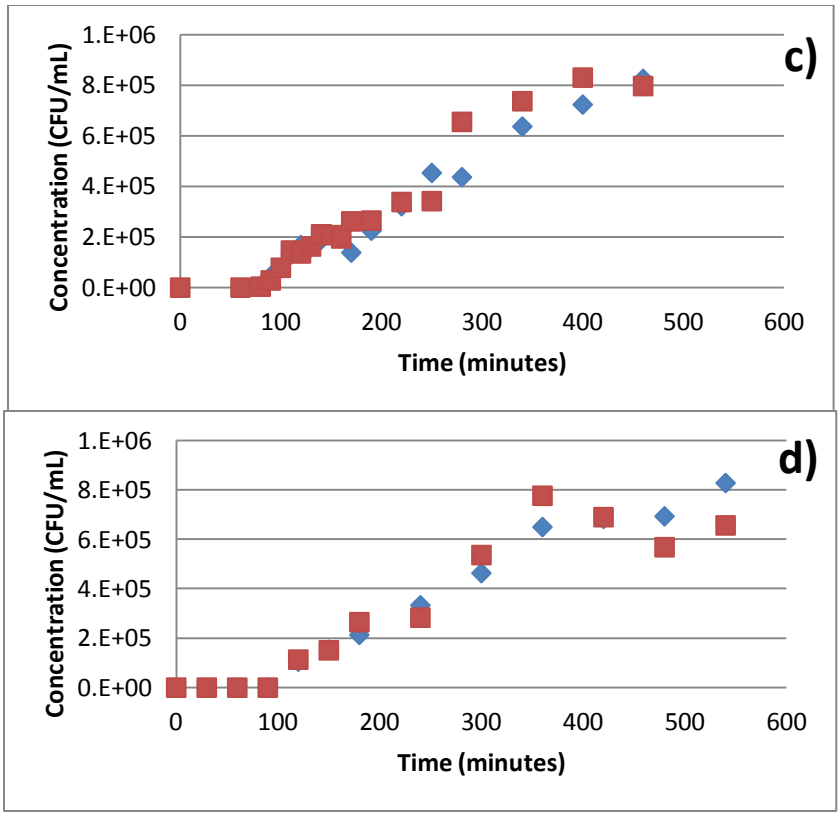

Figure 4.23. Effluent Spore Concentrations of WT 6633 in 10mM KCl from Trials 5 (d) and 6 (e)

Using the final five samples collected, percent removals were calculated along with mean $\log_{10}$ removals (Table 4.18; Figure 4.24). Trial 1 shows a decrease in percent removal, dropping from ~60% to ~20%, while Trial 4 drops from 90% removal to nearer 60% removal. Although showing the same overall trend, trial 5 highlights the difficulty in determining a clear plateau as the five samples collected between 190 and 400 minutes consistently result in ~80% removal of spores. The final five samples of Trial 6 were collected between 300 and 540 minute, and suggest a second plateau beginning potentially as early as 300 minutes. Further experimentation is required to elucidate more precisely spore removal potential under favourable conditions.

Table 4.18 Percent and Mean Log_{10} Removal of WT 6633

[KCl]		10mM							
Trial		1		4		5		6	
Column		A	B	A	B	A	B	A	B
% Removal									
	1	55.27	66.29	95.14	79.43	89.87	79.22	50.24	16.60
	2	56.91	35.37	93.23	83.83	84.27	74.43	30.20	25.90
	3	37.18	27.31	83.24	71.37	70.42	79.38	26.62	38.97
	4	28.63	18.11	73.18	78.32	88.94	60.21	25.55	29.48
	5	18.60	21.39	69.76	60.68	33.07	30.96	11.05	20.36
Average (N=5)		39.32	33.70	82.91	74.73	73.31	64.84	28.73	26.26
SD		16.67	19.37	11.44	9.03	23.80	20.49	14.07	8.66
Mean Log Removal		0.22	0.18	0.77	0.60	0.57	0.45	0.15	0.13

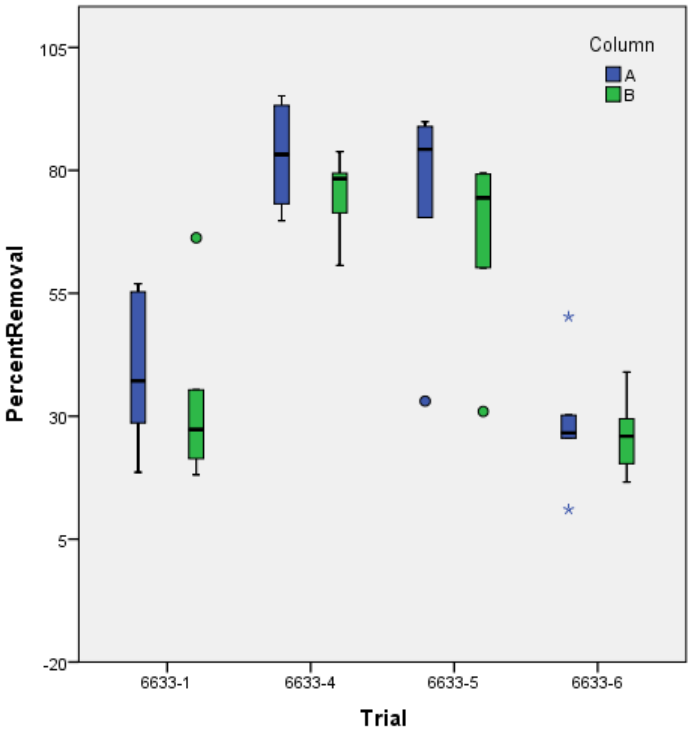

Figure 4.24 Percent Removal of WT 6633 in 10mM KCl

It is possible that the higher frequency of sample collection between 90 and 180 minutes (ten minute intervals), in conjunction with the expected noisiness of concentration calculations using the plate-count method created what appeared to be an early plateau when data were assessed visually. Settling of spores in the feed suspension may also have caused an increase in influent spore concentrations over

time with higher concentrations (~10%) introduce during the final phases of the experiments. Trial 6633-6 (Figure 4.22d) was also conducted to isolate if settling was an issue with reduced sampling during breakthrough and sampling continuing until no feed suspension remained (9 hours). It clearly slows a slowly rising limb, and a plateau is potentially reached at ~6 hours (Table 4.19), indicating Experiment 6 (WT 6633) required an extended sampling schedule beyond 8.5 hours, or increased frequency of sampling after the pseudo steady-state plateau was reached at 6 hours. These data suggest further investigation is generally required for spore removal in favourable conditions. Increased temperatures near the end of the 8 hour experiments conducted in this investigation may also increase removal; Castro and Tufenkji (2004), monitored *E. coli* transport through saturated porous media bench-scale columns and observed higher removal at 22°C in comparison with removal at 11°C. Temperature effects were not investigated here but may warrant further study. Temperature effects may cause bacterial spores to alter or initiate EPS production, or cause changes in steric repulsion as spores respond to their environment and surface proteins change (Kuznar and Elimelech, 2004).

Although a mean 0.32 $\log_{10}$ removal of WT 6633 was observed, Trial 6 suggests that complete breakthrough might be achieved if the full breakthrough curve could be observed. Under the experimental conditions used for this study (Stock volumes, sampling schedule) it was not possible to delineate the full plateau.

Table 4.19 Trial 6633-6 in 10mM KCl

Elapsed Time (m)	Trial 6633-6 Normalized Concentration (C/C0) A	B
0	0	0
0	0	0
30	0	0
60	0.00	0.00
90	0.01	0.10
120	0.10	0.17
150	0.15	0.25
180	0.20	0.27
240	0.32	0.51
300	0.44	0.74
360	0.62	0.66
420	0.66	0.54
480	0.66	0.63
540	0.79	0.71

4.3.2.5 Conclusions

In general, greater spore removal was seen in the higher ionic strength suspension, in agreement with CFT (Table 4.20) (Scholl et al., 1990; Fontes et al., 1991; Gannon et al, 1991b; Mills et al., 1994; Schijven and Hassanizadeh, 2000; Compere et al., 2001; Hsu et al., 2001; Kuznar and Elimelech, 2004; Bradford et al., 2007; Torkzaban et al., 2008). Taking the $\log_{10}$ of the mean percent removal for the final five samples collected during each Experiment, removals ranged between 0.33 $\log_{10}$ and 0.69 $\log_{10,.}$ When grouped, mean removals for both subspecies were identical while LT spores were removed to a greater extent than WT spores, with values of 0.46 and 0.36 $\log_{10}$ respectively, which was contrary to anticipated findings. In unfavourable conditions further investigation employing larger stock volumes and longer sampling schedules is recommended.

Table 4.20 Mean Log $_{10}$ Removal of *Bacillus subtilis* spores in 10mM KCl

***Bacillus* strain**	Mean	N	SD
WT 3610	.44	20	.16
LT W23	.69	15	.14
LT 168	.38	30	.16
WT 6633	.33	40	.09
Grouped Removals			
Strain Type			
WT	.36	50	.14
LT	.46	45	.15
SubSpecies			
subtilis	.40	50	.16
spizizenii	.40	55	.14

4.4 Data Analysis

An ANOVA was performed using the *Bacillus subtilis* percent removal data from the final five samples collected during each trial within each experiment for the four strains in 0.10 and 10mM KCl (Table 4.21). Under the experimental conditions investigated, spore strain did have a significant impact on removal during transport through saturated porous media ($p < 0.05$). p values were higher (0.017) under favourable conditions in comparison with unfavourable conditions (0.000). Significance ($p<0.05$) was also observed in type and subspecies in unfavourable conditions (Table 4.22 and 4.23). Due to the uncertainty of the results presented for 10mM KCl or favourable conditions, it is possible to suggest that the strain of *Bacillus subtilis* has a significant impact on retention in saturated porous media should a more appropriate sampling regime clearly delineate a pseudo steady-state plateau.

Table 4.211 ANOVA on mean *Bacillus subtilis* percent removals by strain

Ionic Strength (mM KCl)		Sum of Squares	Df	Mean Square	F	Sig.
0.10	Between Groups	54367.605	3	18122.535	20.883	.000
	Within Groups	70294.127	81	867.829		
10	Between Groups	8160.316	3	2720.105	3.541	.017
	Within Groups	77585.582	101	768.174		

Table 4.222 ANOVA of mean WT and LT *Bacillus subtilis* percent removals

Ionic Strength (mM KCl)		Sum of Squares	Df	Mean Square	F	Sig.
0.10	Between Groups	22078.012	1	22078.012	17.863	.000
	Within Groups	102583.720	83	1235.948		
10	Between Groups	1945.837	1	1945.837	2.392	.125
	Within Groups	83800.061	103	813.593		

Table 4.233 ANOVA of mean *Bacillus subtilis subtilis* and *spizizenii* percent removals

Ionic Strength (mM KCl)		Sum of Squares	df	Mean Square	F	Sig.
0.10	Between Groups	28455.958	1	28455.958	24.550	.000
	Within Groups	96205.774	83	1159.106		
10	Between Groups	.392	1	.392	.000	.983
	Within Groups	85745.506	103	832.481		

4.5 Sample Handling Errors

4.5.1 Feed Suspension Spore Distribution

An aliquot of 800 µL of 6633 stock suspension was injected into 1500mL of 10mM KCl solution, vortexed, and left stirring overnight in the refrigerator at 4°C, in the same manner as during experiments 15 through 22. While stirring at a manual setting of ~300 rpm, eighteen samples were collected and plated in triplicate to assess spore distribution in the feed suspension (Table 4.5) (Figure B2). No pumping occurred during sample collection.

Table 4.244 Spore Distribution in Feed Suspension with stirring

Sample ID	Dilution	Volume (mL)	Count (CFU)								
Sample Location			Outer Edge			Middle			Centre		
1	10^3	0.2	192	179	170	191	172	217	226	214	192
2	10^3	0.1	97	99	92	104	87	94	87	103	97
3	10^3	0.1	101	93	89	87	69	115	90	87	82
4	10^3	0.2	194	201	169	196	190	193	182	201	188
5	10^3	0.1	91	82	105	98	102	117	78	97	76
6	10^3	0.1	106	103	120	98	101	85	98	104	75

These CFU counts were used to calculate the distribution of spores in the feed suspension during stirring. The resulting feed suspension distribution appears very consistent, excluding the possibility that settling occurred during experimentation.

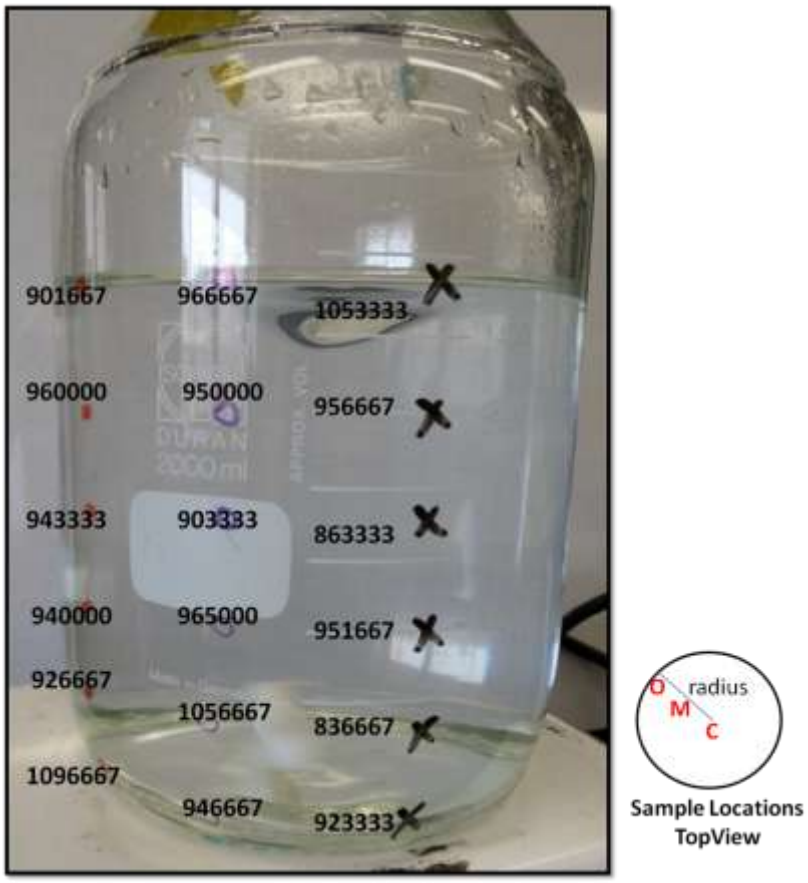

Figure 4.25 Strain 6633 Spore Concentration Distribution in Feed Suspension (CFU/mL) Symbols denote sample collection location: X = Centre, O = Middle, and ● = Outer Edge

The feed suspension concentration calculations from trial 6633-6 suggested that settling might contribute to a small 10% increase in feed suspension concentrations at the end of experimentation, when less than 300mL remained (Table 4.28 and 4.29). This is well within the expected fluctuation of microbial data; enumeration techniques involve losses and result in variable microbial recovery (Nieminski et al., 1995; Emelko et al., 2008) and subsequent calculated concentrations often oscillate about a mean. If stirring was insufficient and settling did occur, this may explain why some effluent concentration calculations were greater than the calculated influent feed suspensions. It does not account for the large increase in filter effluent spore concentrations observed with WT 6633 in 10mM KCl.

Table 4.255 6633-6 Feed Suspension CFU Concentration Calculations

Stock	Dilution	Volume	Count			Mean	CFU/mL
Stock1	1000	0.1	103	77	77	86	856667
Stock2	1000	0.1	111	82	99	97	973333
Stock3	1000	0.1	82	94	88	88	880000
Stock4	1000	0.2	189	171	194	185	923333
Stock5	1000	0.2	157	140	162	153	765000
Stock6	1000	0.2	193	176	213	194	970000
Stock7	1000	0.2	169	177	168	171	856667
Stock8	1000	0.2	178	216	188	194	970000
Stock9	1000	0.1	77	71	73	74	736667
Stock10	1000	0.1	70	81	91	81	806667
Stock11	1000	0.1	91	120	84	98	983333
Stock12	1000	0.1	116	131	104	117	1170000
Stock13	1000	0.1	108	95	107	103	1033333
Stock14	1000	0.1	100	105	114	106	1063333
Stock15	1000	0.1	94	97	103	98	980000

Table 4.266 6633-6 Feed Suspension Concentration Calculations

Time	Average CFU/mL	SD
Initial a.m. C_0	879667	78035
Final C_0	1046000	102404
Evening C	868000	77653
Average (N = 15)	931222	116364

4.5.2 Storage Effects

Spore effluent samples were stored at 4°C until processing. Prior to plating, samples were diluted in 9.0mL of DI with 0.1% Tween80. Storage of filter effluent spore samples in 10mM KCl resulted in an average 6% decrease in spore concentration over a two-week period, with plate counts showing very consistent numbers and variability. Serial dilutions containing spores stored for the same two-week period showed a 33% loss, with counts ranging from 10 to 70% lower than the original concentration calculations performed when those dilutions were initially prepared (Table 4.12). This impact on spore concentrations may be caused by changing background solutions, or an unknown impact of the DI containing the surfactant Tween80. Samples should be processed as soon as possible, or stored in the original background solution. Here,

samples were processed as quickly as possible, within ten days and standard guidelines (ASTM, 2011).

Table 4.27 Storage Effects on Spore Concentrations

	Immediate Preparation					Two-Week Storage: Replated Dilutions				Two-Week Storage: New Dilutions				% Loss	
#	Vol (mL)	Count (CFU)			[CFU/mL]	Vol (mL)	Count (CFU)			[CFU/mL]	Count (CFU)			[CFU/mL]	Replate	New
1	0.1	103	77	77	856667	0.1	53	50	41	480000	74	76	92	806667	44.0	5.8
2	0.1	111	82	99	973333	0.2	129	113	100	570000	191	165	169	875000	41.4	10.1
3	0.1	82	94	88	880000	0.2	105	90	100	491667	159	201	181	901667	44.1	-2.5
4	0.2	189	171	194	923333	0.2	175	168	151	823333	197	181	193	951667	10.8	-3.1
5	0.2	157	140	162	765000	0.2	126	119	112	595000	124	178	116	696667	22.2	8.9
6	0.2	193	176	213	970000	0.2	133	117	134	640000	204	191	171	943333	34.0	2.7
7	0.2	169	177	168	856667	0.1	67	73	68	693333	80	117	105	1006667	19.1	-17.5
8	0.2	178	216	188	970000	0.1	64	78	76	726667	84	86	96	886667	25.1	8.6
9	0.1	77	71	73	736667	0.1	57	65	59	603333	73	97	77	823333	18.1	-11.8
10	0.1	70	81	91	806667	0.1	56	59	49	546667	95	103	69	890000	32.2	-10.3
11	0.1	91	120	84	983333	0.1	73	79	73	750000	101	101	94	986667	23.7	-0.3
12	0.1	116	131	104	1170000	0.1	59	91	86	786667	94	109	101	1013333	32.8	13.4
13	0.1	108	95	107	1033333	0.1	28	37	34	330000	102	144	109	1183333	68.1	-14.5
14	0.1	100	105	114	1063333	0.1	28	34	33	316667	*				70.2	100.0
15	0.1	94	97	103	980000	0.1	76	88	73	790000	84	103	102	963333	19.4	1.7
	All Samples Serially diluted to 10^{-3}													Average	33.7	6.1

*insufficient sample for new dilution

4.5.3 Experimental Conditions

To prevent spore germination, feed suspensions were refrigerated overnight prior to experimentation. Upon initiation of the experiment the temperature of the feed suspensions should have been 4°C, but would increase to approximately room temperature by the end of the 8 hour experiments (Table 4.12). Changing experimental conditions, including changes in feed suspension temperature were identified as a potential contributor to the filter effluent spore concentration increases seen in Experiment 6.

Table 4.28 Experimental Conditions during Trial 6633-6

Time	Temperature (°C)	DO	Conductivity (µS/cm)	pH
7:10am	7.5	11	998	6.97
7:50	10.4	10.9	1074	6.87
9:10	15.3	10.19	1208	6.56
9:40	17	9.76	1255	6.47
10:10	18.4	9.22	1293	6.43
11:10	20.7	8.72	1360	6.35
12:10pm	22.2	8.25	1302	6.33
1:10	23.3	7.72	1434	6.31
2:10	23.8	7.49	1394	6.32
3:10	23.8	7.68	1406	6.3
4:10	23.6	7.43	1421	6.3
5:10	23	7.51	1425	6.34

To investigate this, a probe was inserted into the feed suspension for Experiment 22 (6633-6) to monitor the changing conditions in the feed suspension [temperature (°C), dissolved oxygen (DO), conductivity (µS/cm) and pH] (Table 4.31). As expected, temperature increased gradually but reached room temperature (23°C) at 6 hours, which coincides with the secondary plateau observed with WT 6633 in 10mM KCl. Temperature showed the largest fluctuation. The pH did change (~0.63), but stayed within the range of natural groundwater; DO decreased from 11 to 7 mg/L, while conductivity increased from 1000 to 1425 µS/cm.

Of the parameters measured during this trial, changing temperature seemed the factor most likely contributing to a secondary plateau. Temperature has been seen to increase the size and zeta potential of bacterial spores; higher temperatures have been observed to increase attachment and decrease the absolute value of zeta potential (Gallardo-Moreno et al., 2003; Castro and Tufenkji, 2004). Temperature also affects zeta potential; WT strain 6633 measured -31.8mV in 0.10mM KCl at 25°C, and -45.7 at 4°C. However, a more negative charge (increase in absolute value) should result in decreased attachment, according to results observed by Smets et al. (1999), and supported by CFT. The results of zeta potential measurements presented here showed

an increasing absolute value when ionic strength increased, opposite the trends observed by Kim et al. (2008), and Rice et al. (1996). The zeta potentials of WT strain 6633 showed similar trends and values to those measured for WT strain 3610 (-41.4 and -37.5mV in 10mM KCl respectively. It is more plausible that WT 6633 was unaffected by the parameters measured here and reached a plateau later than the other strains observed.

Chapter 5

Conclusions

Pathogenic microorganisms must be removed from drinking water sources prior to consumption; surrogate (bio)colloids can be used to investigate the pathogen removal capacity of filtration through porous media and assist in decreasing public risk of exposure to waterborne illness caused by *Cryptosporidium* oocyst passage into treated drinking water. To select an appropriate *Cryptosporidium* surrogate, information concerning the transport of the potential surrogate through saturated porous media is required.

The purpose of this investigation was to determine if *Bacillus subtilis* strain selection resulted in quantifiable differences in the transport of *Bacillus subtilis* spores through saturated porous media to such an extent that a standard strain should be recommended for use in drinking water treatment plant process evaluations or riverbank filtration performance demonstrations. The results of these experiments may enable better/more informed use of aerobic endospores for such assessments. Key conclusions of this work are:

1. Under unfavourable conditions, spores of four *Bacillus subtilis* strains showed < 0.22 $\log_{10}$ removal (0 to 0.22$\log_{10}$ range). All four strains were removed to a lesser extent than 1.1µm microspheres (0.66 $\log_{10}$ removal) suggesting spores may be preferentially transported through saturated porous media.
2. Favourable deposition conditions, in high ionic strength solution (10mM KCl), significantly affected the time to plateau. WT spore strain 6633 demonstrated a slowly rising limb in 10mM KCl, potentially reaching a pseudo steady-state plateau at ~6 hours, in contrast to ~170 minutes for all four strains to reach a plateau under unfavourable conditions (0.10mM KCl).
3. The zeta potential of the *Bacillus subtilis* spores was significantly affected by the ionic strength. Strains 3610, W23, and 6633 became more negatively charged; ~31.5mV in 0.10mM KCl to 39.3mV in 10mM KCl. LT strain 168 measured -62.1 in 0.10mM KCl and became less negatively charged in the higher ionic strength (-55.8mV).
4. *Bacillus subtilis* spore removal during transport through saturated porous media may be significantly affected by flow rate, media characteristics, and changing

temperatures. Laboratory handling errors also resulted in settling during experimentation and losses during bacterial assays.

5.1 Recommendations

It is exceptionally difficult to replicate the natural environment in a laboratory setting. Care should be taken when extrapolating micro-scale results to a field- or plant-scale. Based on the experimental results obtained during this investigation, several recommendations can be made for future use of spores in riverbank filtration performance assessments and drinking water treatment plant process evaluations. These are:

- Spore suspensions can be stored up to two weeks, but should remain in the experimental background solution/original suspension. Serial dilution preparations should be performed immediately before spread-plating to prevent changes in concentrations due to the effects of changing background solutions. If possible, perform dilutions using the same solution (ie. 0.10 or 10mM KCl). If a different solution is to be used, the effects of that solution on spores should be investigated and quantified.
- For bench-scale experiments, colloid-containing feed suspensions should be well mixed prior to and during experiments to prevent settling of colloids that may result in changes in influent colloid concentrations. Novel ways of ensuring complete mixing occurs should be investigated.
- Bench-scale column experiments neglect to consider subsurface heterogeneity in water quality, nutrient contents, mineralogy, or sediment/porous media composition. Assignment of removal credits for *Cryptosporidium* based on surrogate transport through the subsurface should be based primarily on field- or full-scale investigations on a site-specific basis.
- The development of non-ideal plug-flow behaviour was visible at the bench scale using a dye tracer. Filter effluent spore concentration variations may be due to the movement of colloids along preferential pathways through porous media due to size exclusion. Flow paths should be assessed prior to experimentation.
- For investigations involving spore spiking (injection of high concentrations of spores), it is recommended that an indigenous environmental strain of aerobic

bacteria be isolated for use to accurately represent local conditions and assessed prior to use.

- The effects of background solution on spore concentrations, germination, and EPS production requires full characterization to adequately assess the suitability of surrogates for pathogen transport investigations.

5.2 Future Work

This investigation contributes to the current state of knowledge on the behaviour of the aerobic endospores of *Bacillus subtilis* during transport through saturated porous media and during laboratory handling. Several of the outcomes of this investigation warrant further investigation to assist in the optimization of spore use during drinking water treatment plant performance demonstrations and riverbank filtration performance assessments. These include:

- The effect of changing temperatures on spore surface characteristics is undocumented. A thorough characterization of EPS and zeta potential would be beneficial to future use of spores.
- Single-temperature experiments will reduce changes in zeta potential and spore surface characteristics during long laboratory investigations. Shorter bench-scale column experiments may not require refrigeration but an assessment of temperature should precede experiments.
- The ability of WT *Bacillus subtilis* strains to form fruiting bodies and increased EPS in high nutrient environments requires furether investigation. EPS production during experimentation should be monitored and, if possible, quantified.
- Hydraulic loading rate should be given adequate consideration when performing evaluations at any scale. Fluctuations in flow at the bench-scale may be an influential factor affecting spore retention in porous media. Spore removal at higher loading rates warrants further investigation.

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Appendix A
Materials and Methods

A.1 Media Sieve Analysis

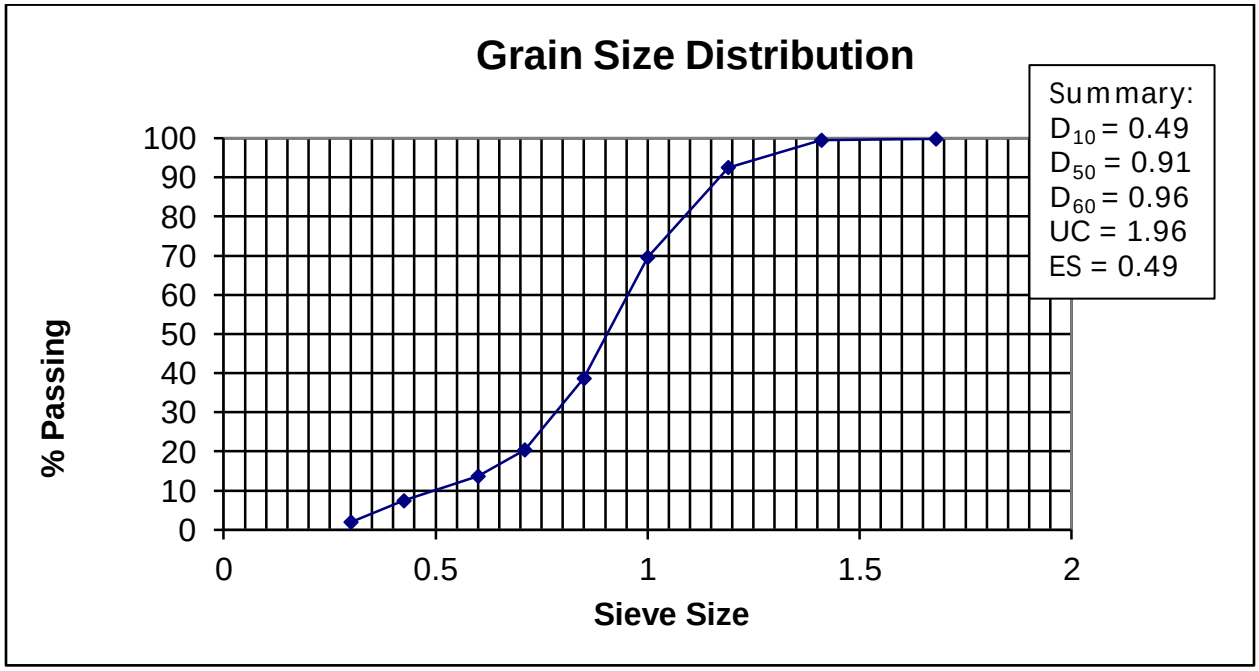

Figure A.1 Grain Size Distribution Analysis for Bulk Sand Sample used in Experiments 1 through 5

Table A.1 Raw Data: Grain Size Analysis for Six-Sample Average from Bulk Sand Sample

Sieve #	Seive size (mm)	weight retained (g)	% retained	Cumulative % retained	Cumulative % passing
12	1.68	0.16	0.001482	0.1482	99.8518
14	1.41	0.36	0.003334	0.4815	99.5185
16	1.19	7.52	0.069636	7.4451	92.5549
18	1	24.78	0.229466	30.3917	69.6083
20	0.85	33.42	0.309473	61.3390	38.6610
25	0.71	19.71	0.182517	79.5907	20.4093
30	0.6	7.27	0.067321	86.3228	13.6772
40	0.425	6.77	0.062691	92.5919	7.4081
50	0.3	5.89	0.054542	98.0461	1.9539
pan		2.11	0.019539	100.0000	0.0000
Total		**107.99**			

Table A.2 Sieve Analysis results: Bulk Sand Sample used in Experiments 1 through 5

Parameter	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
D_{10}	0.4	0.6	0.63	0.45	0.45	0.55
D_{50}	0.84	0.92	0.94	0.94	0.91	0.94
D_{60}	0.88	0.96	0.98	0.98	0.95	0.98
ES	0.4	0.6	0.63	0.45	0.45	0.55
UC	2.2	1.60	1.51	2.18	2.11	1.78

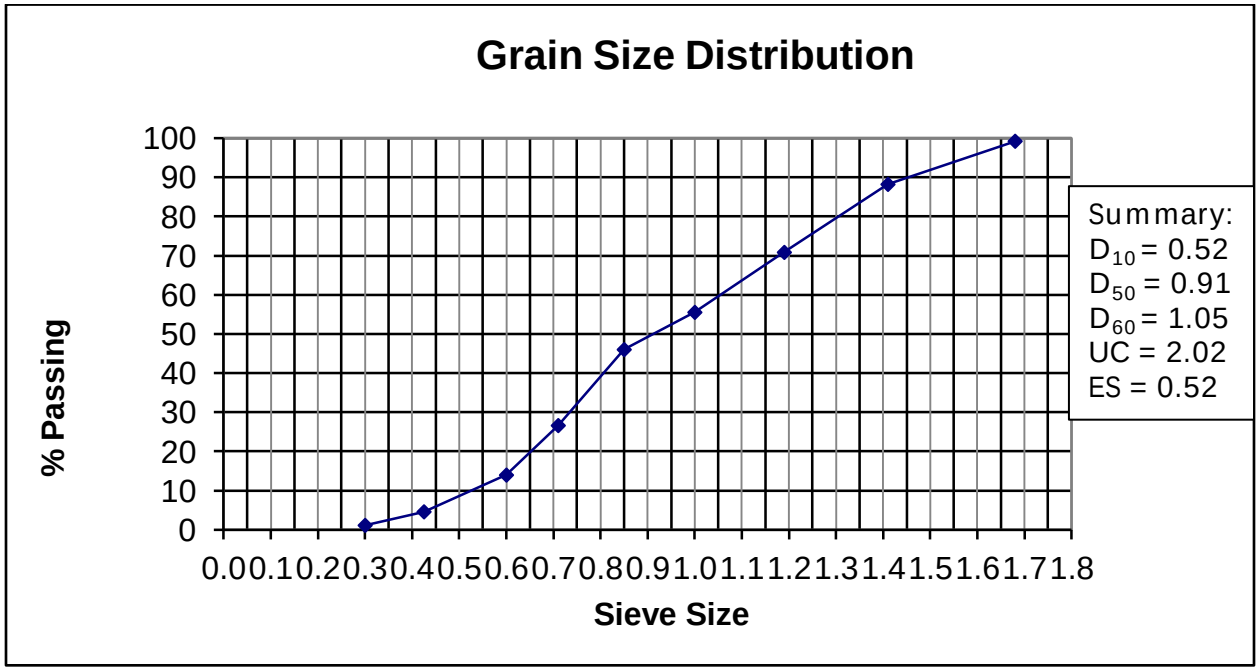

Figure A.2 Sample Grain size Distribution Graph: Experiment 6 Media Sieve Analysis

Table A.3 Experiment 6 Media Sieve Analysis – Sample Raw Data

Sieve #	**Seive size (mm)**	**weight retained (g)**	**% retained**	**Cumulative % retained**	**Cumulative % passing**
12	**1.68**	**7.2**	0.006776	0.677583286	99.32241671
14	1.4	112.3	0.105684	11.2460	88.7540
16	1.19	183.1	0.172313	28.4773	71.5227
18	1.0	148.9	0.140128	42.4901	57.5099
20	0.85	105.6	0.099379	52.4280	47.5720
25	0.71	228.9	0.215415	73.9695	26.0305
30	0.6	124.5	0.117165	85.6861	14.3139
40	0.425	105.1	0.098908	95.5769	4.4231
50	0.3	41.7	0.039243	99.5012	0.4988
pan		12.5	0.011764	100.6776	-0.6776
Total		**1062.6**			

Table A.4 Summary of Sieve Analyses for all Experiments

Experiment #/ Bag #	Parameter	D_{10}/ES	D_{50}	D_{60}	UC
E1 (Bulk)		0.49	0.91	0.96	1.96
E2		0.49	0.91	0.96	1.96
E3		0.49	0.91	0.96	1.96
E4		0.49	0.91	0.96	1.96
E5		0.49	0.91	0.96	1.96
E6		0.49	0.91	0.96	1.96
E7		0.49	0.91	0.96	1.96
E8		0.49	0.91	0.96	1.96
E9 (Bag1)		0.48	0.83	0.95	1.98
E10 (Bag2)		0.49	0.85	0.93	1.90
E11 (Bag3)		0.48	0.88	0.96	2.00
E12 (Bag4)		0.52	0.91	1.05	2.02
E13 (Bag7)		0.5	0.84	0.98	1.96
E14 (Bag5)		0.53	0.88	1.03	1.94
E15 (Bag6)		0.5	0.86	1.01	2.02
E16 (Bag8)		0.52	0.88	1.02	1.96
E17(Bag9)		0.54	0.89	1.03	1.91
E18 (Bag10)		0.52	0.90	1.03	1.98
E19 (Bag11)		0.55	0.91	1.04	1.89
E20 (Bag12)		0.5	0.86	1.0	2.00
E21 (Bag13)		0.54	0.93	1.05	1.81
E22 (Bag14)		0.49	0.86	1.00	2.04
	Average	0.51	0.88	1.00	1.96

A.2 Media Porosity

Table A. 5 Media Porosity

Recorded Weight (grams)	Sample 1	Sample 2
Mass A_{GC}	101.42	101.03
Mass $B_{GC+water}$	202.23	201.88
Mass $C_{GC+dry\ sand}$	268.09	258.41
Mass $D_{GC+wet\text{-}packed\ sand}$	304.48	299.98
V_v (D-C)	36.39	41.57
V_t (B-A)	100.81	100.85
Porosity	0.3609	0.4121

A.3 Loading Rate

Pore volumes were calculated according to the following formula:

Flow

$$Q\frac{m^3}{day} = v\frac{mL}{minute} \times \frac{1L}{1000mL} \times \frac{1m^3}{1000L} \times \frac{60min}{hour} \times \frac{24\ hours}{day}$$

Where:

Q = influent flow rate m^3 / day

and v = pump rate mL / minute

Filter Area (Surface Area of Column media)

$$A = \pi r^2 = \pi(25)^2$$

$$= 1.963\ m^2$$

And $$linear\ velocity = \frac{Q}{filter\ area} = \frac{0.001728\ m^3}{0.001963\ m^2 day}$$

Table A.6 Groundwater Velocity and Loading Rate

Loading Rate				
Column A and B				
Pump Flow Rate (v)	1.2	mL/min	1000	mm in a m
Influent Flow Rate (Q)	0.001728	m^3 /day		
Column Diameter (Ø)	50	mm	1000	L in a m^3
Filter Area	0.0019625	m^2		
Linear Velocity	8.81E-01	m^3	1000	mL in a L
~Porosity	0.39			
Groundwater Velocity	2.3	m/day		
Loading Rate	0.1	m/hour		

A.4 Theoretical Pore Volume Calculations

Table A. 7 Column Pore Volume

Typical Flow		
Column Diameter (Ø)	50	mm
Column Length (L)	150	mm
Volume in Column	2.94E-04	m^3
~Porosity	0.39	
PV (L)	0.1148	L
PV (mL)	114.8	mL
Flow Rate	1.2	mL/min
Time to center of advective flux	95.7	Min

Table A.8 Theoretical Pore Volumes at Observed Flow Rates

Flow Rate (mL/min)	**Theoretical PV (mL)**
1.0	114.8
1.1	104.4
1.2	95.7
1.3	88.3
1.4	82.0

A.5 Nutrient Exhaustion Sporulation method

Sporulation Induction using 2xSG Agar (Nicholson and Setlow, 1990):

1. Combine the following in 1000mL of DI water:
    - Nutrient Broth 16.0g
    - KCl 2.0g
    - Bacteriological Agar 17.0g
    - $MgSO_4$ 0.5g
    - 50% (w/v) glucose 1g/L final
2. Adjust the pH to 7.0 (with what?) and then autoclave for 15 minutes at 121°C
3. Prepare 100x stock in 1000mL DI water (use 10mL per L of agar)
    - $Ca(NO_3)_2*4H_20$ 16.4g
    - $MnCl_2*4h_20$ 0.95g
    - $FeSO_4$ 0.015G

Appendix B
Data

Bromide Tracer Tests

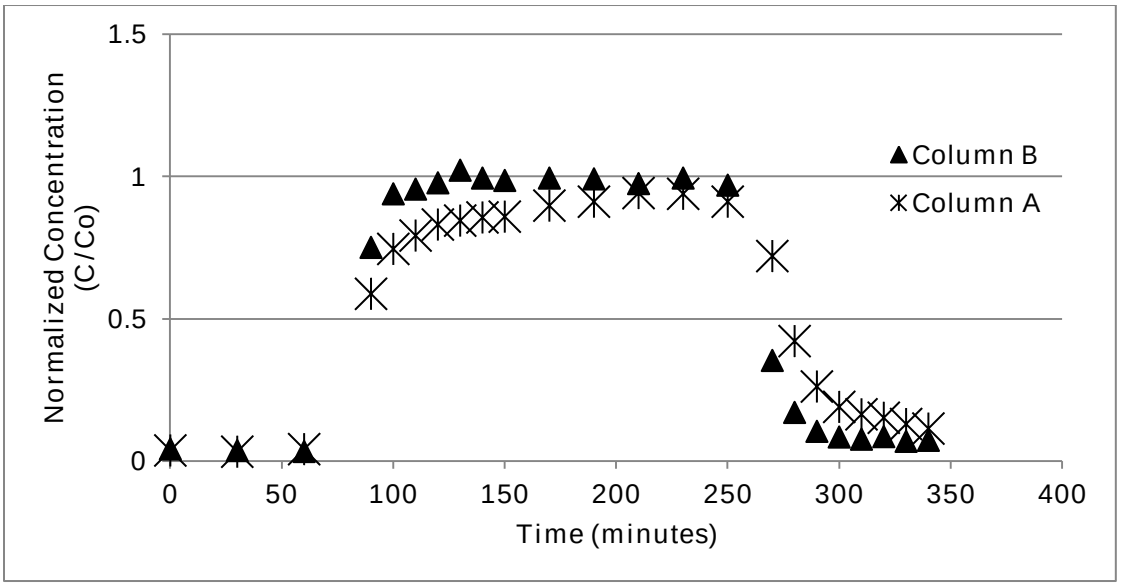

Figure B. 1 Experiment 3 Bromide Tracer – 0.1mM KCl

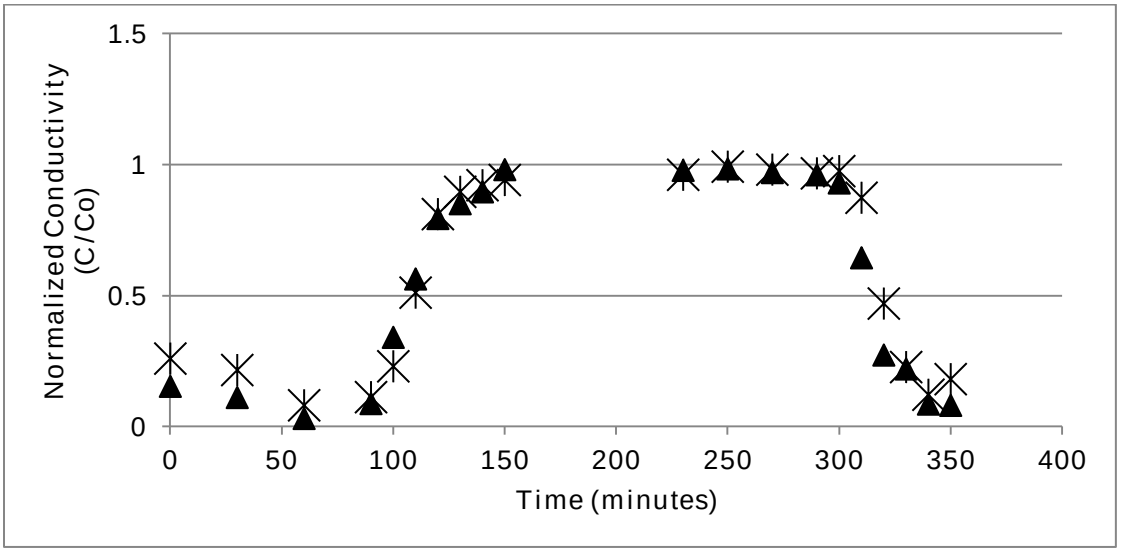

Figure B. 2 Experiment 6 Bromide Tracer – 0.10 mM KCl

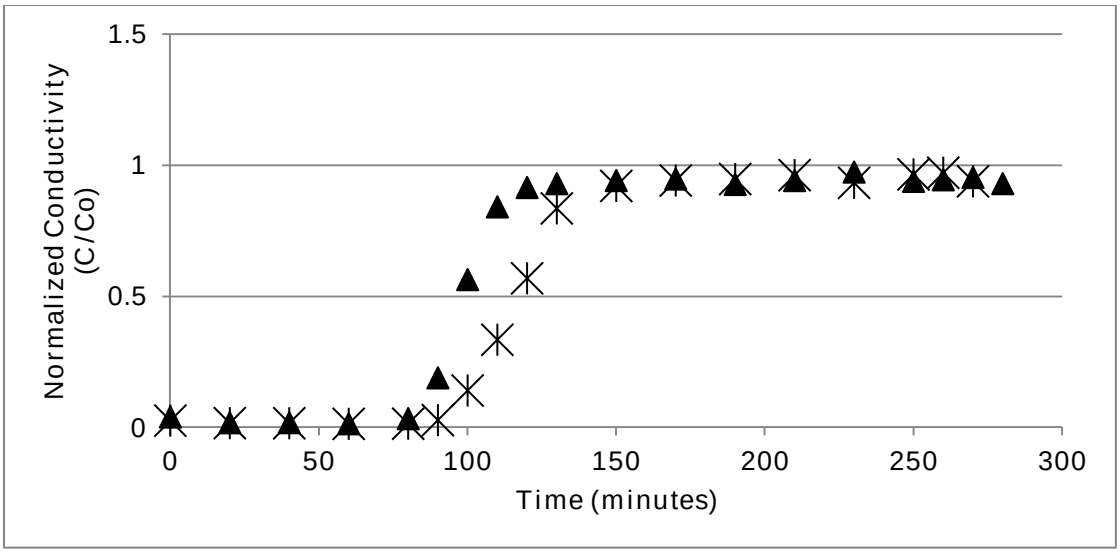

Figure B. 3 Experiment 4 Bromide Tracer – 10mM KC l

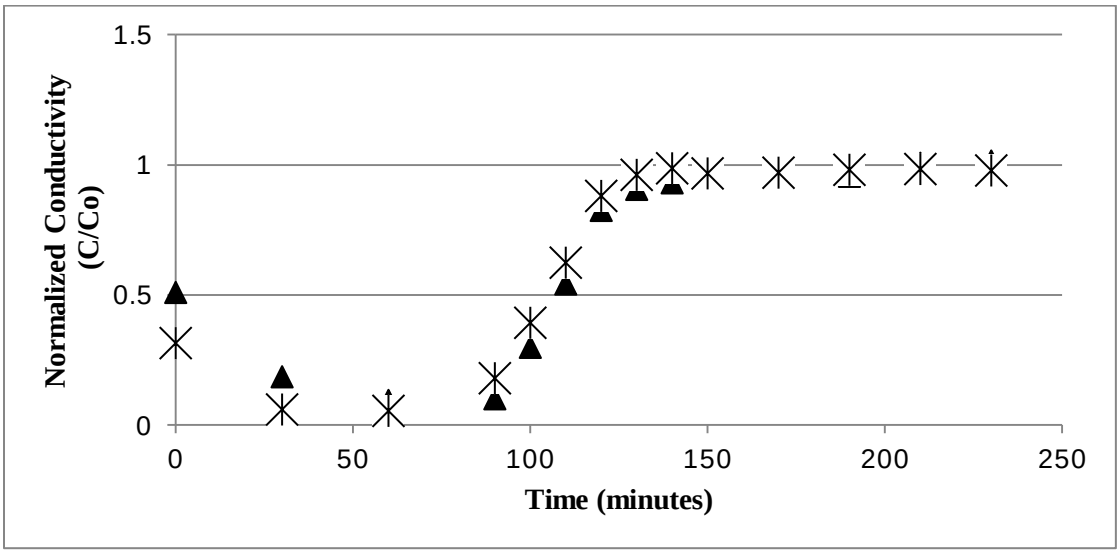

Figure B. 4 Experiment 5 Bromide Tracer – 10mM KCl

Table B. 1 Sample Bromide Breakthrough Curve Raw Data

Real Time	Sample		Elapsed Time		Column A					Column B				
	Duration	3			Flow	vol passed	P.V.	µS	Norm	Flow	vol passed	P.V.	µS	Norm
					ml/min	mL		pm	C/C0	ml/min	mL		pm	C/C0
	No	Interval	mins	hrs										
18:40	0	30	0	0.0	1.4	0	0	16.3	0.047731	1.4	0	0	31	0.090776
19:10	1	30	30	0.5	1.4	42	0.42	14.1	0.041288	1.3	39	0.39	29.2	0.085505
19:40	2	30	60	1.0	1.4	84	0.84	12.2	0.035725	1.4	81	0.81	16.3	0.047731
20:10	3	30	90	1.5	1.3	123	1.23	50.5	0.147877	1.4	123	1.23	36.8	0.10776
20:20	4	10	100	1.7	1.3	136	1.36	99.7	0.291947	1.4	137	1.37	87.4	0.25593
20:30	5	10	110	1.8	1.4	150	1.5	161.4	0.472621	1.3	150	1.5	186.6	0.546413
20:40	6	10	120	2.0	1.3	163	1.63	220	0.644217	1.4	164	1.64	251	0.734993
20:50	7	10	130	2.2	1.3	176	1.76	287	0.84041	1.4	178	1.78	302	0.884334
21:00	8	10	140	2.3	1.3	189	1.89	313	0.916545	1.3	191	1.91	313	0.916545
21:10	9	20	160	2.7	1.3	215	2.15	321	0.939971	1.3	217	2.17	330	0.966325
21:30	10	40	200	3.3	1.3	267	2.67	313	0.916545	1.4	273	2.73	328	0.960469
22:10	11	20	220	3.7	1.3	293	2.93	328	0.960469	1.3	299	2.99	325	0.951684
22:30	12	20	240	4.0	1.3	319	3.19	327	0.95754	1.4	327	3.27	327	0.95754
22:50	13	10	250	4.2	1.5	334	3.34	321	0.939971	1.4	341	3.41	327	0.95754
23:00	14	10	260	4.3	1.4	348	3.48	317	0.928258	1.4	355	3.55	317	0.928258
23:10	15	10	270	4.5	1.5	363	3.63	294	0.860908	1.4	369	3.69	304	0.89019
23:20	16	10	280	4.7	1.4	377	3.77	207	0.606149	1.3	382	3.82	196	0.573939
23:30	17	10	290	4.8	1.3	390	3.9	121.5	0.355783	1.4	396	3.96	92.8	0.271742
23:40	18	10	300	5.0	1.4	404	4.04	60.3	0.176574	1.4	410	4.1	48.7	0.142606
23:50	19	10	310	5.2	1.3	417	4.17	42.9	0.125622	1.4	424	4.24	37.8	0.110688
0:00	20	10	320	5.3	1.3	430	4.3	41.9	0.122694	1.4	438	4.38	36.5	0.106881

*Feed change occurred at sample 10 (green)

Stock Concentration Calculations

Table B. 2 Sample Stock Concentration Calculation: W23 - 5

		Average Initial	352589.7							
		Average Final	264566.7							
		Total Average	314318.8							
		Average with Exclusions	290285.7							
sample #	**Dilution**	**Volume**	**Count (CFU)**					**Average Count**	**[CFU/mL]**	**Exclude Low**
1	100000	0.1	0	0	1			0.3	333333.3	
	10000	0.1	8					8.0	800000.0	
	1000	0.1	40					40.0	400000.0	400000.0
	1000	0.2	95	75	71	64	91	79.2	396000.0	396000.0
2	1000	0.2	72	60	63	80	75	70.0	350000.0	350000.0
3	1000	0.2	66	75	65	77	58	68.2	341000.0	341000.0
4	1000	0.2	74	89	75	67	59	72.8	364000.0	364000.0
5	1000	0.2	78	77	77	70	74	75.2	376000.0	376000.0
11	1000	0.2	47	35	29			37.0	185000.0	185000.0
12	1000	0.2	48	62	59			56.3	281666.7	281666.7
13	1000	0.2	52	57	54			54.3	271666.7	271666.7
14	1000	0.2	42	51	59			50.7	253333.3	253333.3
15	1000	0.2	49	51	39			46.3	231666.7	231666.7
6	1000	0.2	50	57	66	49	46	53.6	268000.0	268000.0
7	1000	0.2	70	40	64	52	64	58.0	290000.0	290000.0
8	1000	0.2	50	70	45	60	52	55.4	277000.0	277000.0
9	1000	0.2	66	46	73	55	67	61.4	307000.0	307000.0
10	1000	0.2	65	61	52	65	59	60.4	302000.0	302000.0
16	1000	0.2	42	27	41			36.7	183333.3	183333.3
17	1000	0.2	55	62	60			59.0	295000.0	295000.0
18	1000	0.2	53	57	53			54.3	271666.7	271666.7
19	1000	0.2	56	43	34			44.3	221666.7	221666.7
20	1000	0.2	47	42	49			46.0	230000.0	230000.0

Electrophoretic Mobility

Table B. 3 Zeta Potential: Assessment of concentration and heat-shock effects

168	Heatshocked							
Concentration	**10^2**	**10^3**	**10^4**	**10^8**	**10^2**	**10^3**	**10^4**	**10^8**
Read1	-8.5	-8.7	-7.7	-29.8	-6.7	-8.7	-13.0	-29.5
Read2	-8.9	-13.5	-20.8	-30.6	-10.0	-10.3	-11.9	-30.0
Read3	-8.0	-12.9	-7.2	-29.8	-10.0	-12.3	-11.1	-29.8
Average	-8.5	-11.7	-11.9	-30.1	-8.9	-10.4	-12.0	-29.8

Error messages were received that the concentration of these samples (10^2-10^4) might be too low (Figure B2). Full concentration stock suspension samples with concentrations higher than 10,000 (10^7 to 10^{10}) were used for reported electrophoretic potential and size measurements. Microsphere suspensions were prepared aiming for 10^{10} particles/mL (NR) and 10^8 particles/mL (YG) to account for the larger size of the YG MS.

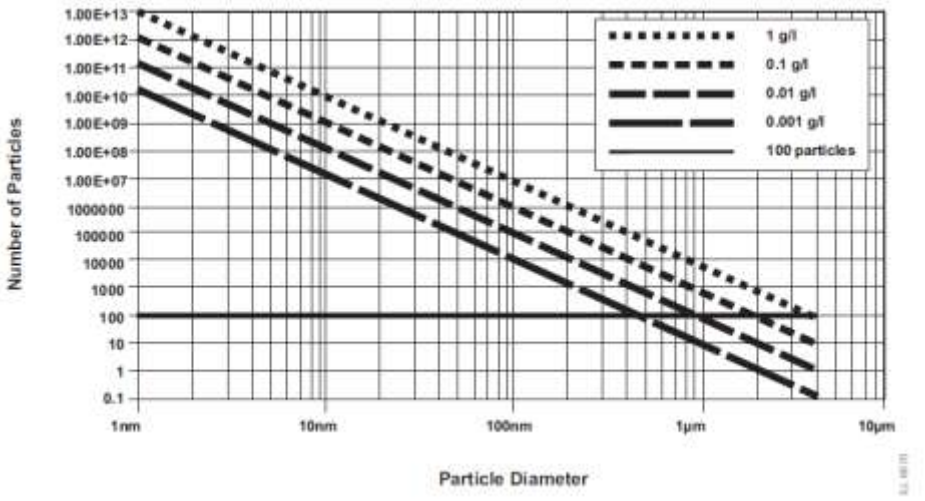

Figure B. 5 Particle Number Requirements for ZetaSizerNano (Malvern, 2004)

Table B4. SPSS Coding

SPSS Code Key

Colloid		Colloid Type	Subspecies	Trials/Exp	Column Labels	Volume		IS KCl	
1	NR MS 1.1	3	n/a	1	A	0	1500	0	0.10mM
2	YG MS 4.5	3	n/a	1	B	1	2000	1	10mM
3	3610	1 WT	1 *subtilis*	4					
4	W23	2 LT	2 *spizizenii*	5					
5	168	2 LT	1 *subtilis*	5					
6	6633	1 WT	2 *spizizenii*	6					

Table B5. Effluent Calculations for Microsphere Experiments (E1 NR MS 1.1; E16 YG MS 4.5)

NR MS 1.1			YG MS 4.5					
		Feed = 862083					Feed = 103222	
ms/mL	**C/C0**	**Log Removal**	**ms/mL**		**C/C0**		**Log Removal**	
251000	0.291155	0.53587536	36	31	0.000345	0.000297	3.462625	3.527565
144000	0.167037	0.777186589	36.5	25.5	0.000349	0.000244	3.456634	3.612387
250000	0.289995	0.537609072	47	34	0.00045	0.000326	3.346829	3.487448
359000	0.416433	0.380454632	46	35	0.00044	0.000335	3.356169	3.474859
184500	0.214017	0.669552711	42	38	0.000402	0.000364	3.395678	3.439144
263000	0.305075	0.515593333						
314000	0.364234	0.438619433						
239000	0.277235	0.55715118						

Table B. 4 Normalized Concentration of WT *spizizenii* 6633 in 10mM KCl

	Trial 6633-1		**Trial 6633-4**		**Trial 6633-5**	
	[Feed]	1013500	[Feed]	1653177	[Feed]	1042889
	Normalized Concentration C/C0					
Elapsed Time (m)	A	B	A	B	A	B
0	0	0	0	0	0	0
60	0	0	0	0	0	0.00
80	0.01	0.00	0.00	0.00	0.00	0.00
90	0.04	0.03	0.01	0.00	0.00	0.00
100	0.08	0.08	0.02	0.01	0.02	0.02
110	0.13	0.14	0.04	0.03	0.06	0.05
120	0.17	0.13	0.06	0.05	0.07	0.08
130	0.16	0.16	0.07	0.08	0.10	0.11
140	0.19	0.21	0.08	0.12	0.16	0.12
150	0.21	0.20	0.09	0.09	0.19	0.18
160	0.20	0.19	0.07	0.13	0.09	0.24
170	0.14	0.26	0.08	0.14	0.09	0.28
190	0.22	0.26	0.08	0.21	0.13	0.19
220	0.32	0.33	0.09	0.21	0.10	0.21
250	0.45	0.34	nd	nd	0.16	0.26
280	0.43	0.65	0.05	0.16	nd	nd
310	nd	nd	nd	nd	0.30	0.21
340	0.63	0.73	0.07	0.29	nd	nd
400	0.71	0.82	0.17	0.22	0.11	nd
430	nd	nd	nd	nd	nd	0.40
460	0.81	0.79	0.27	0.39	0.67	0.69
490	nd	nd	nd	nd	nd	0.10
520	nd	nd	0.30	nd	nd	nd

Table B6. *Bacillus subtilis* spore removal raw data and removal calculations

3610-1					[Feed]	912857
	[CFU 3610] CFU/mL		Normalized		Removal	
Time (m)	**A**	**B**	**C/Co A**	**C/Co B**	**LogA**	**LogB**
			Trial 1			
0	1	0	1.10E-06		5.96	
30	18	0	1.97E-05		4.71	
60	16		0.00		4.76	
90	199	511	0.00	0.00	3.66	3.25
100						
110		95500		0.10		0.98
120						
130		450000		0.49		0.31
140	257333		0.28		0.55	
150	504667	805000	0.55	0.88	0.26	0.05
160	706667		0.77		0.11	
170	803333	892000	0.88	0.98	0.06	0.01
180	873333		0.96		0.02	
210	800000	830000	0.88	0.91	0.06	0.04
270	705000		0.77		0.11	
330	860000	900000	0.94	0.99	0.03	0.01
360	790000		0.87		0.06	
420	1086667	1120000	1.19	1.23	-0.08	-0.09
480	826667		0.91		0.04	
510	836667	1050000	0.92	1.15	0.04	-0.06
540	1075000		1.18		-0.07	
570	1195000	1200000	1.31	1.31	-0.12	-0.12
600	630000		0.69		0.16	
610	310000	805000	0.34	0.88	0.47	0.05

3610-2					[Feed]	170611
	[CFU 3610] CFU/mL		Normalized		Removal	
Time (m)	**A**	**B**	**C/Co A**	**C/Co B**	**LogA**	**LogB**
			Trial 2			
0	6.7	1.7	0.00	0.00	4.41	5.00
30	11.7	1.7	0.00	0.00	4.16	5.00
60	1.7	0	0.00		5.00	
90	1588	0	0.01		2.03	
100	6100	368	0.04	0.00	1.45	2.67
110	20333	2400	0.12	0.01	0.92	1.85
120	38333	4600	0.22	0.03	0.65	1.57
130	66750	27000	0.39	0.16	0.41	0.80
140	87333	104000	0.51	0.61	0.29	0.21
150	144333	52400	0.85	0.31	0.07	0.51
160	152666	69800	0.89	0.41	0.05	0.39
170	39000	75966	0.23	0.45	0.64	0.35
180	56333	98666	0.33	0.58	0.48	0.24
190	171666	68666	1.01	0.40	0.00	0.40
210	145000	105200	0.85	0.62	0.07	0.21
230	140000	118333	0.82	0.69	0.09	0.16
250	166666	136666	0.98	0.80	0.01	0.10
270	233333	140000	1.37	0.82	-0.14	0.09
300	143333	140000	0.84	0.82	0.08	0.09
330	98333	110000	0.58	0.64	0.24	0.19
390	161666	140000	0.95	0.82	0.02	0.09
450	94666	176666	0.55	1.04	0.26	-0.02

3610-3					[Feed]	182199
	[CFU 3610] CFU/mL		Normalized		Removal	
Time (m)	A	B	C/Co A	C/Co B	LogA	LogB
			Trial 3			
0	5	0	0.00		4.56	
30	0	0	0.00			
60	0	0	0.00			
80	820	3.3	0.00		2.35	
90	7666	183	0.04	0.00	1.38	3.00
100	22333	3466.7	0.12	0.02	0.91	1.72
110	56800	19666	0.31	0.11	0.51	0.97
120	68000	51000	0.37	0.28	0.43	0.55
130	87333	68333	0.48	0.38	0.32	0.43
140	160999	73666	0.88	0.40	0.05	0.39
150	140000	79666	0.77	0.44	0.11	0.36
170	160000	75000	0.88	0.41	0.06	0.39
190	148333	70000	0.81	0.38	0.09	0.42
210	170333	66333	0.93	0.36	0.03	0.44
240	124666	80333	0.68	0.44	0.16	0.36
270	138666	95000	0.76	0.52	0.12	0.28
300	116333	60666	0.64	0.33	0.19	0.48
330	90000	85666	0.49	0.47	0.31	0.33
390	195666	86333	1.07	0.47	-0.03	0.32
450	64333	79666	0.35	0.44	0.45	0.36
510	209000	81000	1.15	0.44	-0.06	0.35

3610-3					[Feed]	151481
	[CFU 3610] CFU/mL		Normalized		Removal	
Time (m)	A	B	C/Co A	C/Co B	LogA	LogB
			Trial 4			
0	0	1.7	0	1.1E-05		4.95
30	3.3	0	2.2E-05	0	4.66	
60	3.3	0	2.2E-05	0	4.66	
70	2170	6.7	0.01	0.00	1.84	4.35
80	8000	380	0.05	0.00	1.28	2.60
90	15333	3000	0.10	0.02	0.99	1.70
100	29333	9433	0.19	0.06	0.71	1.21
110	26333	16500	0.17	0.11	0.76	0.96
120	27333	17333	0.18	0.11	0.74	0.94
130	30666	27500	0.20	0.18	0.69	0.74
140	30666	27000	0.20	0.18	0.69	0.75
150	34000	29500	0.22	0.19	0.65	0.71
160	28166	28000	0.19	0.18	0.73	0.73
180	16000	13833	0.11	0.09	0.98	1.04
200	22333	34500	0.15	0.23	0.83	0.64
220	31333	30000	0.21	0.20	0.68	0.70
280	19666	31000	0.13	0.20	0.89	0.69
340	18666	28666	0.12	0.19	0.91	0.72
400	23666	23000	0.16	0.15	0.81	0.82
430	15000	23000	0.10	0.15	1.00	0.82
460	15000	20333	0.10	0.13	1.00	0.87

W23-1					[Feed]	365385
	[CFU 3610] CFU/mL		Normalized		Removal	
Time (m)	A	B	C/Co A	C/Co B	LogA	LogB
			Trial 1			
0	0	0		0		
20	0	0		0		
40	0	0		0		
60	0	0	0	0		
70	0	0	0	0		
80	0	0	0	0		
90	105	0	0.00	0.00	3.54	
100	1225	33.5	0.00	0.00	2.47	4.04
110	10950	677	0.03	0.00	1.52	2.73
120	36850	6900	0.10	0.02	1.00	1.72
130	71500	26117	0.20	0.07	0.71	1.15
140	118667	55500	0.32	0.15	0.49	0.82
150	141333	104333	0.39	0.29	0.41	0.54
160	155000	128333	0.42	0.35	0.37	0.45
170	160500	171500	0.44	0.47	0.36	0.33
190	179000	143333	0.49	0.39	0.31	0.41
210	172000	216333	0.47	0.59	0.33	0.23
230	198000	220000	0.54	0.60	0.27	0.22
260		212333		0.58		0.24
290	206667	213333	0.57	0.58	0.25	0.23
320	157000	238166	0.43	0.65	0.37	0.19
350	160667	213000	0.44	0.58	0.36	0.23
410	191333	225666	0.52	0.62	0.28	0.21

W23-2					[Feed]	912857
	[CFU 3610] CFU/mL		Normalized		Removal	
Time (m)	A	B	C/Co A	C/Co B	LogA	LogB
			Trial 2			
0	0	0	0	0		
30	3	0	7E-06	0	5.16	
60	0	2	0	4.6E-06		5.33
90	23	10	5.3E-05	2.3E-05	4.27	4.63
100	430	453	0.00	0.00	3.00	2.98
110	1303	3408	0.00	0.01	2.52	2.10
120	6367	13083	0.01	0.03	1.83	1.52
130	12017		0.03		1.55	
140	18667	36500	0.04	0.08	1.36	1.07
150	17000	46167	0.04	0.11	1.40	0.97
160	18900	21358	0.04	0.05	1.36	1.30
170		47000		0.11		0.96
180	25333	46000	0.06	0.11	1.23	0.97
200	23500	33833	0.05	0.08	1.26	1.11
220	18500	39167	0.04	0.09	1.37	1.04
240	18683		0.04		1.36	
260	27333	32333	0.06	0.08	1.20	1.12
280	21833	44000	0.05	0.10	1.30	0.99
310	30167	43000	0.07	0.10	1.15	1.00
340	23667	43500	0.05	0.10	1.26	1.00
400	20833		0.05		1.32	
460	13333	31000	0.03	0.07	1.51	1.14

W23-3					[Feed]	1039445
	[CFU 3610] CFU/mL		Normalized		Removal	
Time (m)	A	B	C/Co A	C/Co B	LogA	LogB
			Trial 3			
0	0	0	0	0		
30	0	5	0	4.8E-06		5.32
60	0	0	0	0		
90	16383	1600	0.02	0.00	1.80	2.81
100	57833	10067	0.06	0.01	1.25	2.01
110	120333	34000	0.12	0.03	0.94	1.49
120	227666	61667	0.22	0.06	0.66	1.23
130	278000		0.27		0.57	
140	380000		0.37		0.44	
150	400000		0.38		0.41	
160	425000		0.41		0.39	
180	546667		0.53		0.28	
200	523333		0.50		0.30	
220	500000		0.48		0.32	
240	510000		0.49		0.31	
270	436667		0.42		0.38	
300	480000		0.46		0.34	
330	490000		0.47		0.33	
390	433333		0.42		0.38	
450	543333		0.52		0.28	
510	486667		0.47		0.33	

W23-4				[Feed]	379856
	[CFU 3610] CFU/mL		Normalized	Removal	
Time (m)	A	B	C/Co A	C/Co B	LogA	LogB
			Trial 4			
0	0		0.00	0.00		
20	0		0.00	0.00		
40	0		0.00	0.00		
60	3.3		0.00	0.00	5.06	
80	3750		0.01	0.00	2.01	
90	35749		0.09	0.00	1.03	
100	58666		0.15	0.00	0.81	
110				0.00		
120	233166		0.61	0.00	0.21	
130	288333		0.76	0.00	0.12	
140	286777		0.75	0.00	0.12	
150	257166		0.68	0.00	0.17	
160	308500		0.81	0.00	0.09	
170	320166		0.84	0.00	0.07	
180	235833		0.62	0.00	0.21	
200	205500		0.54	0.00	0.27	
220	282000		0.74	0.00	0.13	
240	366666		0.97	0.00	0.02	
270	455000		1.20	0.00	-0.08	
300	360000		0.95	0.00	0.02	
330	180416		0.47	0.00	0.32	
390	329333		0.87	0.00	0.06	
450	378333		1.00	0.00	0.00	

W23-5					[Feed]	352589
	[CFU 3610] CFU/mL		Normalized		Removal	
Time (m)	A	B	C/Co A	C/Co B	LogA	LogB
			Trial 5			
0	0	0	0	0		
30	3	5	0.00	0.00	5.07	4.85
60	23	0	0.00	0.00	4.19	
90	17016	8633	0.05	0.02	1.32	1.61
100	82666	42833	0.23	0.12	0.63	0.92
110	130000	64333	0.37	0.18	0.43	0.74
120	202600	182833	0.57	0.52	0.24	0.29
130	219583	162916	0.62	0.46	0.21	0.34
140	257333	209999	0.73	0.60	0.14	0.23
150	301000	338250	0.85	0.96	0.07	0.02
160	246666	202833	0.70	0.58	0.16	0.24
170	404000	353333	1.15	1.00	-0.06	0.00
180	318000	357500	0.90	1.01	0.04	-0.01
190	404000	260000	1.15	0.74	-0.06	0.13
210	413000	375000	1.17	1.06	-0.07	-0.03
230	309666	376666	0.88	1.07	0.06	-0.03
250	472000	313333	1.34	0.89	-0.13	0.05
270	381000	286666	1.08	0.81	-0.03	0.09
300	393000	443333	1.11	1.26	-0.05	-0.10
330	474000	501666	1.34	1.42	-0.13	-0.15
360	418000	313333	1.19	0.89	-0.07	0.05
390	534000	418333	1.51	1.19	-0.18	-0.07
420	484000	300000	1.37	0.85	-0.14	0.07

168-1					[Feed]	246933
	[CFU 3610] CFU/mL		Normalized		Removal	
Time (m)	**A**	**B**	**C/Co A**	**C/Co B**	**LogA**	**LogB**
			Trial 1			
0	0	12		0.00		4.31
60	0	2		0.00		5.09
80	0	7		0.00		4.55
90	22.5	475	0.00	0.00	4.04	2.72
100	1238	5767	0.01	0.02	2.30	1.63
110	13433	36500	0.05	0.15	1.26	0.83
120	49677	118667	0.20	0.48	0.70	0.32
130	148667	225000	0.60	0.91	0.22	0.04
140	129666	333333	0.53	1.35	0.28	-0.13
150	218333	341667	0.88	1.38	0.05	-0.14
160	340000	380000	1.38	1.54	-0.14	-0.19
170	188333	350000	0.76	1.42	0.12	-0.15
190	215000	388333	0.87	1.57	0.06	-0.20
220	220000	376667	0.89	1.53	0.05	-0.18
250	278333	303333	1.13	1.23	-0.05	-0.09
310	311667	530000	1.26	2.15	-0.10	-0.33
370	238333	500000	0.97	2.02	0.02	-0.31
430	235000	490000	0.95	1.98	0.02	-0.30
520	400000	533333	1.62	2.16	-0.21	-0.33

168-2					[Feed]	3857
	[CFU 3610] CFU/mL		Normalized		Removal	
Time (m)	**A**	**B**	**C/Co A**	**C/Co B**	**LogA**	**LogB**
			Trial 2			
0	0	0				
30	0	0				
60	0	0				
80	20	18.3	0.01	0.00	2.29	2.32
90	245	86.7	0.06	0.02	1.20	1.65
100	573.3	170	0.15	0.04	0.83	1.36
110	623.3	386.7	0.16	0.10	0.79	1.00
120	770	220	0.20	0.06	0.70	1.24
130	756.7	298.3	0.20	0.08	0.71	1.11
140	646.7	356.7	0.17	0.09	0.78	1.03
150	796.7	261.7	0.21	0.07	0.68	1.17
170	696.7	356.7	0.18	0.09	0.74	1.03
190	780	440	0.20	0.11	0.69	0.94
210	608.3	388.3	0.16	0.10	0.80	1.00
240	566.7	276.7	0.15	0.07	0.83	1.14
270	493.3	251.7	0.13	0.07	0.89	1.19
330	336.7	261.7	0.09	0.07	1.06	1.17
390	510	176.7	0.13	0.05	0.88	1.34
450	308.3	153.3	0.08	0.04	1.10	1.40
510	295		0.07648		1.11643	

168-3					[Feed]	1050500
	[CFU 3610] CFU/mL		Normalized		Removal	
Time (m)	**A**	**B**	**C/Co A**	**C/Co B**	**LogA**	**LogB**
			Trial 3			
0	0	0				
30	0	0				
60	0	0				
80	0.85	0	0.00		6.09	
90	82	43	0.00	0.00	4.11	4.39
100	1318.3	1420	0.00	0.00	2.90	2.87
110	9433.3	12433	0.01	0.01	2.05	1.93
120	36333.3	36067	0.03	0.03	1.46	1.46
130	78166.7	88167	0.07	0.08	1.13	1.08
140	146666.7	133333	0.14	0.13	0.86	0.90
150	206666.6	177750	0.20	0.17	0.71	0.77
160	235666.7	197333	0.22	0.19	0.65	0.73
170	305000	193333	0.29	0.18	0.54	0.74
190	456666.7	70667	0.43	0.07	0.36	1.17
210	465000	65000	0.44	0.06	0.35	1.21
240	635000	391000	0.60	0.37	0.22	0.43
270	918333.3	815000	0.87	0.78	0.06	0.11
330	966666.7	976667	0.92	0.93	0.04	0.03
420	631666.7	1028333	0.60	0.98	0.22	0.01
510	458333.3	723333	0.44	0.69	0.36	0.16

168-4					[Feed]	813288
	[CFU 3610] CFU/mL		Normalized		Removal	
Time (m)	**A**	**B**	**C/Co A**	**C/Co B**	**LogA**	**LogB**
			Trial 4			
0	13.3	3.3	0.00	0.00	4.83	5.43
30	0	3.3		0.00		5.43
60	1.7	8.3	0.00	0.00	5.72	5.03
70	158.3	833.3	0.00	0.00	3.75	3.03
80	6400	12533.3	0.01	0.01	2.14	1.85
90	34166.7	79833.3	0.04	0.09	1.42	1.05
100	66333.3	308500	0.07	0.35	1.13	0.46
110	185833.4	241666	0.21	0.27	0.68	0.57
120	284000	591666	0.32	0.66	0.50	0.18
130	603333	723333	0.68	0.81	0.17	0.09
140	845000	816666	0.95	0.92	0.02	0.04
150	908333	950000	1.02	1.07	-0.01	-0.03
160	950000	853333	1.07	0.96	-0.03	0.02
180	673333.3	955000	0.76	1.07	0.12	-0.03
200	1133333	1153333	1.27	1.29	-0.10	-0.11
220	1203333	970000	1.35	1.09	-0.13	-0.04
280	1096666	926666	1.23	1.04	-0.09	-0.02
340	923333.3	896666	1.04	1.01	-0.02	0.00
400	1010000	956666	1.13	1.07	-0.05	-0.03
430	1063333	1056666	1.19	1.19	-0.08	-0.07
460	961666.7	1080000	1.08	1.21	-0.03	-0.08

168-5					[Feed]	651167
	[CFU 3610] CFU/mL		Normalized		Removal	
Time (m)	**A**	**B**	**C/Co A**	**C/Co B**	**LogA**	**LogB**
			Trial 5			
0	16	2	0.00	0.00	4.61	5.51
30	10	0	0.00		4.81	
60	7	3	0.00	0.00	4.97	5.34
75	0	0				
90	2	2	0.00	0.00	5.51	5.51
100	85	18	0.00	0.00	3.88	4.56
110	1680	587	0.00	0.00	2.59	3.05
120	10750	5067	0.02	0.01	1.78	2.11
130	25167	32500	0.04	0.05	1.41	1.30
140	45167	47667	0.07	0.07	1.16	1.14
150	63667	63667	0.10	0.10	1.01	1.01
160	83333	95333	0.13	0.15	0.89	0.83
170	100000	111333	0.15	0.17	0.81	0.77
190	105000	115750	0.16	0.18	0.79	0.75
210	130000	133333	0.20	0.20	0.70	0.69
240	135000	187333	0.21	0.29	0.68	0.54
270	161667	125000	0.25	0.19	0.61	0.72
330	253333	258333	0.39	0.40	0.41	0.40
390	288333	335000	0.44	0.51	0.35	0.29
450	441667	445000	0.68	0.68	0.17	0.17
510	316250	403116	0.49	0.62	0.31	0.21

6633-1					[Feed]	1013500
	[CFU 3610] CFU/mL		Normalized		Removal	
Time (m)	**A**	**B**	**C/Co A**	**C/Co B**	**LogA**	**LogB**
			Trial 1			
0	0	0	0	0		
60	0	0	0	0		
80	7583	3933	0.01	0.00	2.13	2.41
90	45500	28400	0.04	0.03	1.35	1.55
100	77333	78667	0.08	0.08	1.12	1.11
110	130333	146667	0.13	0.14	0.89	0.84
120	168500	135000	0.17	0.13	0.78	0.88
130	159000	162667	0.16	0.16	0.80	0.79
140	189000	210667	0.19	0.21	0.73	0.68
150	210166	207333	0.21	0.20	0.68	0.69
160	201667	194333	0.20	0.19	0.70	0.72
170	138333	261667	0.14	0.26	0.86	0.59
190	225000	265000	0.22	0.26	0.65	0.58
220	321667	338333	0.32	0.33	0.50	0.48
250	453333	341667	0.45	0.34	0.35	0.47
280	436667	655000	0.43	0.65	0.37	0.19
340	636667	736667	0.63	0.73	0.20	0.14
400	723333	830000	0.71	0.82	0.15	0.09
460	825000	796667	0.81	0.79	0.09	0.10

6633-2					[Feed]	1027000
	[CFU 3610] CFU/mL		Normalized		Removal	
Time (m)	**A**	**B**	**C/Co A**	**C/Co B**	**LogA**	**LogB**
			Trial 2			
0	0	0	0	0		
60	2	0	0.00	0.00	5.71	
90	945	15	0.00	0.00	3.04	4.84
100	690	9367	0.00	0.01	3.17	2.04
110	41167	5533	0.04	0.01	1.40	2.27
120	60667	23367	0.06	0.02	1.23	1.64
130	256667	50000	0.25	0.05	0.60	1.31
140	250000	92667	0.24	0.09	0.61	1.04
150	606667	236667	0.59	0.23	0.23	0.64
160	338333	508333	0.33	0.49	0.48	0.31
170	693333	558333	0.68	0.54	0.17	0.26
190	653333	780000	0.64	0.76	0.20	0.12
210	690000	803333	0.67	0.78	0.17	0.11
240	673333	656667	0.66	0.64	0.18	0.19
300	673333	723333	0.66	0.70	0.18	0.15
360	676667	673333	0.66	0.66	0.18	0.18
420	570000	655000	0.56	0.64	0.26	0.20
480	600000	603333	0.58	0.59	0.23	0.23
540	613333	533333	0.60	0.52	0.22	0.28

6633-3					[Feed]	2752778
	[CFU 3610] CFU/mL		Normalized		Removal	
Time (m)	A	B	C/Co A	C/Co B	LogA	LogB
			Trial 2			
0	0	0	0	0		
60	0	0	0	0		
75	0	0	0	0		
90	903	32	0.00	0.00	3.48	4.93
105	25200	6833	0.01	0.00	2.04	2.61
115	338166	34667	0.12	0.01	0.91	1.90
125	656667	214333	0.24	0.08	0.62	1.11
135	851667	520000	0.31	0.19	0.51	0.72
145	1213333	803333	0.44	0.29	0.36	0.53
155	1253333	910000	0.46	0.33	0.34	0.48
165	1436667	1060000	0.52	0.39	0.28	0.41
175	1420000	1150000	0.52	0.42	0.29	0.38
185	1520000	1220000	0.55	0.44	0.26	0.35
195	1373333	1223333	0.50	0.44	0.30	0.35
225	1600000	1236667	0.58	0.45	0.24	0.35
285	2133333	1666667	0.77	0.61	0.11	0.22
345	1546667	1370000	0.56	0.50	0.25	0.30
405	1646667	1533333	0.60	0.56	0.22	0.25
465	1586667	1530000	0.58	0.56	0.24	0.26
525	1763333	1456667	0.64	0.53	0.19	0.28

6633-4					[Feed]	1653177
	[CFU 3610] CFU/mL		Normalized		Removal	
Time (m)	**A**	**B**	**C/Co A**	**C/Co B**	**LogA**	**LogB**
			Trial 4			
0	2	0	0.00	0.00	5.92	
60	10	0	0.00	0.00	5.22	
80	1260	15	0.00	0.00	3.12	5.04
90	8300	1053	0.01	0.00	2.30	3.20
100	32500	11100	0.02	0.01	1.71	2.17
110	64333	57333	0.04	0.03	1.41	1.46
120	97333	81667	0.06	0.05	1.23	1.31
130	107667	131000	0.07	0.08	1.19	1.10
140	126333	197333	0.08	0.12	1.12	0.92
150	148000	142667	0.09	0.09	1.05	1.06
160	108000	211500	0.07	0.13	1.18	0.89
170	130667	238333	0.08	0.14	1.10	0.84
190	128000	350000	0.08	0.21	1.11	0.67
220	155166	340000	0.09	0.21	1.03	0.69
280	80333	267333	0.05	0.16	1.31	0.79
340	112000	473333	0.07	0.29	1.17	0.54
400	277000	358333	0.17	0.22	0.78	0.66
460	443333	650000	0.27	0.39	0.57	0.41
520	500000		0.30	0.00	0.52	

6633-5					[Feed]	1042889
	[CFU 3610] CFU/mL		Normalized		Removal	
Time (m)	**A**	**B**	**C/Co A**	**C/Co B**	**LogA**	**LogB**
			Trial 5			
0	0	0	0	0		
60	0	15	0.00	0.00		4.84
80	893	67	0.00	0.00	3.07	4.19
90	4900	1327	0.00	0.00	2.33	2.90
100	19567	16500	0.02	0.02	1.73	1.80
110	58167	51833	0.06	0.05	1.25	1.30
120	73667	81000	0.07	0.08	1.15	1.11
130	101333	118667	0.10	0.11	1.01	0.94
140	165833	126000	0.16	0.12	0.80	0.92
150	201000	185800	0.19	0.18	0.72	0.75
160	91333	255000	0.09	0.24	1.06	0.61
170	96667	293333	0.09	0.28	1.03	0.55
190	140333	198333	0.13	0.19	0.87	0.72
220	105667	216667	0.10	0.21	0.99	0.68
250	164000	266667	0.16	0.26	0.80	0.59
310	308500	215000	0.30	0.21	0.53	0.69
400	115333		0.11		0.96	
430		415000	0.00	0.40		0.40
460	698000	720000	0.67	0.69	0.17	0.16
490		102000	0.00	0.10		1.01

6633-6					[Feed]	931222
	[CFU 3610] CFU/mL		Normalized		Removal	
Time (m)	**A**	**B**	**C/Co A**	**C/Co B**	**LogA**	**LogB**
			Trial 6			
0	0	0	0	0		
30	0	0	0	0		
60	3.333333	3.333333	3.2E-06	3.2E-06	5.49536	5.49536
90	11383.33	104667	0.01092	0.10036	1.96197	0.99843
120	105333.3	173833.5	0.101	0.16668	0.99567	0.7781
150	153666.7	265000	0.14735	0.2541	0.83166	0.59499
180	213333.3	283333.3	0.20456	0.27168	0.68918	0.56594
240	333333.3	536666.7	0.31962	0.5146	0.49536	0.28853
300	463333.3	776666.7	0.44428	0.74473	0.35234	0.128
360	650000	690000	0.62327	0.66162	0.20532	0.17939
420	683333.3	568333.3	0.65523	0.54496	0.18361	0.26363
480	693333.3	656666.7	0.66482	0.62966	0.1773	0.20089
540	828333.3	741666.7	0.79427	0.71117	0.10003	0.14803

Key Code

Colloid	[KCL]
3610	0.1mM
W23	10mM
168	
6633	